BRITISH JOURNAL OF CANCER

VOL. XIII DECEMBER, 1959 NO. 4

THE OCCURRENCE OF CANCER IN HUSBANDS AND WIVES

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Received for publication October 3, 1959

THIS investigation was undertaken as an attempt to answer the question "How important are *common* domestic environmental factors in the aetiology of cancers?"

There is a large literature devoted to studies of cancer in families. In these studies the influence of genetic and environmental factors are usually almost inextricably interwoven. (An exception to this generality is the study of cancer occurring in twins separated at birth, but, even here, we have a common ante-natal environment which is of at least theoretical importance.)

As we cannot conveniently remove environmental factors in studies of family or household cancer, one must consider how genetic factors might be eliminated, leaving only those of environment. By genetic factors here we refer to inherited factors shared by members of the same family rather than to genetic factors common to a community. This means that we have to study the occurrence of cancer in members of the same households who are not blood relatives. Material for such a study would be found by examining the causes of death of husbands and their wives. This is the approach used in the present investigation.

Hypotheses tested in the investigation

If one made the hypothesis that gastric carcinomata often had their causation in the consumption of "over-cracked" cooking fats, a deduction might be made which could be tested by observation. Dr. Percy Stocks has pointed out (personal communication) that, very likely, even cancer of a particular site may be produced by various causes. This deduction might run—"Husbands and wives tend to eat food that has been cooked by a common method X. Therefore, if cooking habit X (e.g. cracked fat) is aetiologic in many cases of stomach cancer, one would expect to find that, where wives had this disease, their husbands would tend to be at statistical risk of suffering from it also, eventually". The same argument, in a broader or more general form, would run thus—"Whatever factors a. b. c. . . . in the domestic environment, common to husband and wife, that cause cancer in one partner, may be expected, when other factors are equal, to produce cancer in the other partner". As a corollary to this one could formulate. "If cancer generally (or specific cancers) tend to be associated in husband and wife

more than would be expected on the basis of chance (as determined by controlled studies) it is likely that there is a common domestic environmental factor (or factors) operative in the aetiology of their neoplasms ". Conversely, to formulate the negative proposition, one might assert it as likely that " If cancer in the husbands of women who had died of cancer is no commoner than cancer in the husbands of women who did not die from cancer, then it is unlikely that common domestic factors are important carcinogenic agents."

These hypothetical considerations were put to the test of observation in the manner now described.

MATERIAL

The study was made from death certificates. Copies of these were available for the following districts for years given in brackets :—

City of Winchester, 18 years (1939 to 1956 inclusive).
Urban District of Merton and Morden, 18 years (1939 to 1956 inclusive).
Borough of Eastleigh, 19 years (1938 to 1956 inclusive).
Urban District of Banstead, 19 years (1938 to 1956 inclusive).
Municipal Borough of Gosport, 3 years (1954 to 1956 inclusive).
Metropolitan Borough of Fulham, 20 years (1937 to 1956 inclusive).
Deaths of residents dying both in and outside the districts were used.

METHOD AND PROCEDURE

Every death certificate of a female in the above towns during the period (in most places fifteen to twenty years) was examined, and all widows dying from cancer, who totalled 1869, had small cards prepared giving name, date of death age, diagnosis in international classification of causes of death, serial number, address, husband's Christian names and occupation.

1869 Control widows were then selected from among those who died of non-neoplastic conditions according to the following criteria : Each cancer widow (the words " cancer widow " mean a widow who herself died from cancer) was matched with a control widow (*a*) who died during the same year, if possible in the same month, or at least the same quarter : (*b*) who died at the same age exactly, or at least within two years of the same age : (*c*) who came from the same district.

Exceptions to these rules were extremely few, and occurred when either (i) the number of deaths in the district under survey was small, or (ii) the age on death of the " cancer " widow was so low that a " control " widow could not be found.

The same facts as were noted for the cancer widows were noted for the control widows on similar cards. Much care was taken in selecting the " controls ", and, subject to the factors mentioned in (i) and (ii), the highest degree of adherence to rules (*a*) and (*b*) was obtained by a general review of widows dying from neoplastic conditions, to see the possibilities of accurate matching, before selection for matching took place.

After a final re-check of matching pairs in respect of age and date of death, the two sets of cards were inserted in racks, in alphabetical order, commencing with 1956, and, as each year's cards were racked, the death records for that year were

read aloud by one worker, while the other worker scanned the racks in the following way: The surname of each male entry whose age at death was consistent with his having been married before death (i.e. approximately 18 and over) was read out. When a surname was read which occurred among the cards "racked", the husband's Christian names and occupation and the addresses of both widow and male entry were compared; where all three tallied it was assumed that the two entries were husband and wife; comparison of ages at year of husband's death was used as a final check, and if all factors pointed to a reasonable presumption of "husband" and "wife", the following particulars of the husband were entered on the reverse of the widow's card: Date of death, age, diagnosis and serial number. Cases occurred in which one or more of the necessary factors were not known in either the widow's or husband's case, e.g. in Banstead the only address of the one partner was often given as Banstead Mental Hospital; in such cases, if the Christian names and occupation of the male entry were comparable on both card and entry, and age at death was also consistent with marriage, then the same assumption was made as when addresses also tallied. If husband's occupation was not given on either card or entry, Christian names and age were taken as basis of a reasonable assumption of marriage; but if neither Christian names nor occupation were given in both cases, so that at least one of the two factors could be compared, age was not considered sufficient basis for any assumption of marriage, and no action was taken.

The search for the husbands of the 1869 widows who died from cancer revealed 417 pairs (i.e. husband and wife) in which the causes of death were known. When the search for the husbands of the 1869 "non-cancer widows" (i.e. widows who did not die from cancer) was completed, there was available a total of 455 pairs (each pair consisted of a widow who did not die from cancer and her husband, the cause of whose death was, again, known).

The information on the small cards was coded for cause of death in accordance with the 4 digit code of International Classification of Diseases. Occupation and social class were also coded according to the Registrar General's Classification of Occupations and Social Class Grouping. The cards were given pair numbers for matching husband and wife. Power Samas cards were then punched and verified for each of the 1744 individuals in the investigation to show death register entry number, place and date of death, sex, age, occupation (of husband) social class, cause of death (in parts 1 and 2 of the certificate) cause of death (in parts 1 and 2 of the certificate) of spouse, pair number and whether cancer widow or control widow or husband of cancer widow or husband of non-cancer widow.

Comparability of final material

Although cancer widows and controls used in the original search were carefully matched, it was considered essential to examine the comparability of the final material. The purpose of this verification was to reduce the possibility that unknown selection factors had operated during the search for the husbands to upset the comparability of the two groups.

Age comparability of cancer and non-cancer widows

Table I shows the numbers of cancer widows in five-year age groups, together with a column showing what percentage of all the 417 cancer widows is formed by those in each group. The table also shows the corresponding figures for non-

cancer widows, while Fig. 1 shows the age distribution by these percentages for the two groups of widows. It will be seen that the age composition of the two groups is closely comparable, so that failure to find the husbands of cancer widows of different ages has been paralleled by compensating failures in the search for the husbands of non-cancer widows in such a way that the matching of age groups has not been upset.

TABLE I.—*Age Composition of the Two Groups of Widows*

		40–	45–	50–	55–	60–	65–	70–	75–	80–	85–	90–	95–	All ages
Cancer widows	No.	5	5	16	36	50	64	72	85	51	29	4	—	417
	%	1·2	1·2	3·8	8·6	12·0	15·4	17·3	20·3	12·2	7·0	1·0	—	100
Non-cancer widows	No.	3	6	12	27	51	80	90	97	48	31	10	—	455
	%	0·7	1·3	2·6	5·9	11·2	17·6	19·8	21·3	10·6	6·8	2·2	—	100

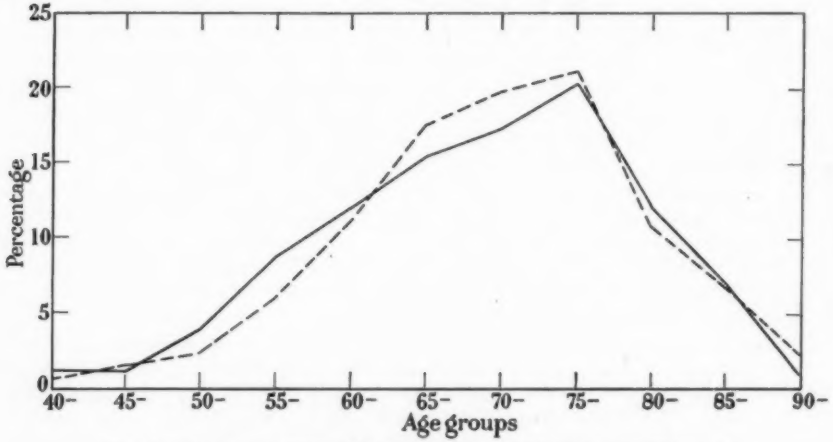

FIG. 1.—Age composition of the two groups of widows as percentages of each group (from Table I).

———— Cancer widows 417.
– – – – – – – – Non-cancer widows 455.

Social class comparability of cancer and non-cancer widows

Table II compares the social class distribution of the two groups of widows. Fig. 2 shows the percentage of these two groups in the different social classes from which it will be seen that the two groups were very similar in this respect.

TABLE II.—*Social Class Composition of the Two Groups of Widows*

		Social Class						
		1	2	3	4	5	Not stated	Total
Cancer widows	No.	16	64	213	51	60	13	417
	%	3·8	15·4	51	12	14·5	3·3	100
Non-cancer widows	No.	21	68	235	65	58	8	455
	%	4·6	15	51·6	14·3	12·7	1·8	100

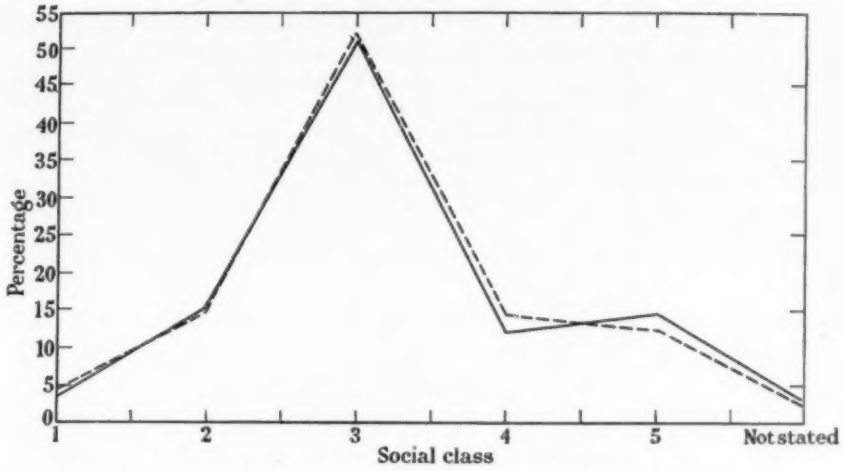

FIG. 2.—Social class composition of the two groups of widows as percentages of each group (from Table II).

———— Cancer widows 417.
– – – – – – – Non-cancer widows 455.

Comparability of cancer and non-cancer widows in respect to district

Table III and Fig. 3 show that the proportion of cancer widows and non-cancer widows coming from different districts was closely comparable.

TABLE III.—*Places of Residence of the Two Groups of Widows*

		Gosport	Eastleigh	Win-chester	Banstead	Merton and Morden	Fulham	All districts
Cancer widows	No.	9	56	34	38	94	186	417
	%	2·2	13·4	8·3	9·1	22·5	44·5	100
Non-cancer widows	No.	13	63	32	27	109	211	455
	%	3·0	13·9	7·0	5·9	24·0	46·2	100

We conclude that we have then two groups of widows, one of which died from cancer, the other of which did not, which were comparable in respect of the age distribution of the group, social class, place of residence and year of death.

The husbands—age composition

Table IV shows (columns 1 and 2) the age distribution (in five-year age groups) of the husbands of the cancer widows. It also shows the percentage in each age group of the total of (417) husbands of cancer widows. The corresponding figures for the 455 husbands of non-cancer widows is shown in columns 3 and 4 of the same table.

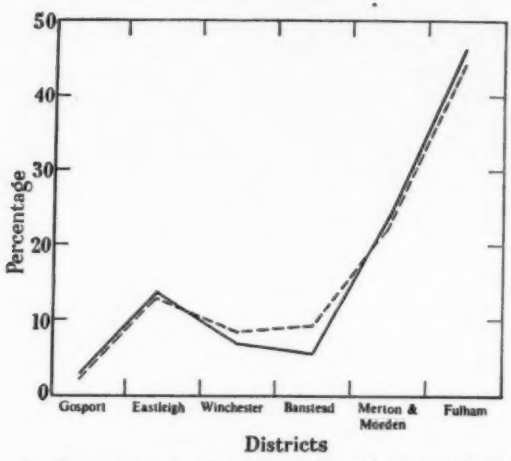

FIG. 3.—Percentage of each group of widows in the six districts.

– – – – – – – Cancer widows 417.
——————— Non-cancer widows 455.

TABLE IV.—*Age Distribution of the Husbands of the Two Groups of Widows*

Col. No.		30–	35–	40–	45–	50–	55–	60–	65–	70–	75–	80–	85–	90–	Not stated	All ages
	Husbands of Cancer Widows															
1	No.	—	—	5	12	26	39	52	77	89	56	50	9	2	—	417
2	%	—	—	1·2	2·9	6·2	9·3	12·6	18·4	21·3	13·4	12·0	2·2	0·5	—	100
	Husbands of Non-cancer Widows															
3	No.	1	2	4	6	25	28	65	86	83	86	47	13	8	1	455
4	%	0·2	0·4	0·9	1·3	5·5	6·2	14·3	18·9	18·2	18·9	10·3	2·9	1·8	0·2	100

The husbands—social class

The social classes of the husbands is, by definition, the same as that of their wives (see Table II and Fig. 2).

The husbands—Geographical distribution

The district of residence of the husbands at the time of death is the same as that of their wives (see Table III and Fig. 3).

RESULTS

1. *Cancer in the husbands of non-cancer (i.e. control) widows*

Of 455 husbands of non-cancer widows, 94 men died from cancer, i.e. 20·6 per cent.

2. *Cancer in the husbands of cancer widows*

Of 417 husbands of cancer widows, 83 men died from cancer, i.e. 20 per cent.

Further information about husbands dying from cancer

(a) *Age distribution.*—Table V shows the age distribution of the 94 cancer deaths among the total of 455 dead husbands of non-cancer widows, together with the distribution of these deaths as percentages of the deaths in each age group of men who were husbands of non-cancer widows. It also shows the age distribution of the 83 cancer deaths among the total of 417 dead husbands of cancer widows, together with the distribution of these deaths as percentages of the deaths in each age group of men who were husbands of cancer widows.

Table VI compares the age distribution of the cancer cases in the husbands of the cancer widows and the non-cancer widows. They are seen to be similar.

TABLE V.—*Age Distribution of Cancer Deaths Among the Two Groups of Husbands*

	Husbands of cancer widows			Husbands of non-cancer widows		
Age groups	All husbands	Husbands who died from cancer	Per cent	All husbands	Husbands who died from cancer	Per cent
0–54	43	10	23·3	38	10	26·4
55–64	91	18	19·8	93	19	20·5
65–74	166	38	22·9	169	40	23·6
75+	117	17	14·5	155	25	16·2
All ages	417	83	20·0	455	94	20·6

TABLE VI.—*Age Distribution of Cancer Cases in the Two Groups of Husbands*

	Husbands of cancer widows who themselves died of cancer		Husbands of non-cancer widows who themselves died of cancer	
Age groups	Number	Per cent	Number	Per cent
0–54	10	12·1	10	10·6
55–64	18	21·7	19	20·2
65–74	38	45·8	40	42·6
75+	17	20·4	25	26·6
All ages	83	100	94	100

(b) *Social class.*—Table VII shows that the distribution of the cancer cases among the social classes follows that of the group of husbands of which they form part and is generally similar in both groups. Nevertheless, a relatively

TABLE VII.—*Social Class Distribution of Cancer Cases in the Two Groups of Husbands*

	Social Class						
	1	2	3	4	5	Not stated	Total
Cancer cases among Husbands of Cancer Widows							
No.	4	12	42	11	12	2	83
%	4·8	14·6	50·4	13·3	14·5	2·4	100
Cancer cases among Husbands of Non-cancer Widows							
No.	5	9	48	19	12	1	94
%	5·3	9·6	51·0	20·3	12·7	1·1	100

larger number of cases fall into Group 2 and a small number into Group 4 amongst the pairs where both husband and wife died from cancer than amongst the pairs where the husband only died from cancer.

(c) *Geographical distribution of cases of cancer in the husbands.*—Table VIII shows the distribution amongst the 6 areas in the survey of the 83 cases of cancer in the husbands of cancer widows and the percentage in each area. It also shows the distribution amongst the same areas of the 94 cases of cancer in the husbands of non-cancer widows. Comparison of the two groups shows that the percentages were very similar in all areas except Gosport where the numbers were very small. It is also seen that in Banstead the percentage of cancer in both groups, i.e. 31·5 per cent and 33·3 per cent, was higher than the total percentages (20 per cent and 20·6 per cent respectively).

TABLE VIII.—*Numbers and Percentages of Husbands in Each Area who Died From Cancer, for Each of the Two Groups of Widows*

Districts	Cancer widows			Non-cancer widows		
	Total	With cancer husbands	Per cent	Total	With cancer husbands	Per cent
Gosport	9	1	11·0	13	4	30·8
Eastleigh	56	14	25.0	63	11	17·5
Winchester	34	5	14·7	32	5	15·6
Banstead	38	12	31·5	27	9	33·3
Merton and Morden	94	21	22·3	109	26	23·8
Fulham	186	30	16·1	211	39	18·5
All districts	417	83	20·0	455	94	20·6

Association of Specific Sites of Neoplasm in Husband and their Wives

The above remarks apply to cancer of all sites taken together. An examination was also made to see whether the husbands of women dying from growths of particular sites were themselves more likely than might be expected by chance (i.e. more than the husbands of wives who did not die from cancer would be) to die from the same particular site growth, or cancer of some other site. An account of this is given in the Appendix and Table IX.

DISCUSSION AND COMMENTS ON RESULTS

It will be seen from the above results that the percentage of husbands of cancer widows who also died of cancer did not differ from the percentage of husbands of non-cancer widows who died from cancer. It appears then that the occurrence of cancer in the wives was not linked with the occurrence of cancer in their husbands who pre-deceased them. To put matters simply, if a wife died from cancer there was no more chance that her husband would die from cancer than if he were the husband of someone else who did not die from cancer. Therefore, there is no evidence, from this investigation (at this time and place), that habits common to husband and wife have detectable importance in causation of cancers from which both could suffer.

TABLE IX

Diagnosis	All cancer widows	Husband and wife with same growth		Non-cancer widows' husbands who died from growth of these sites	
		No.	%	No.	% (of 455)
Tongue	1	—	..	..	..
Floor of mouth	1	—	..	..	..
Other parts of mouth	1	—	..	..	..
Hypopharynx	1	—	..	..	..
Oesophagus	6	—	..	..	..
Stomach	65	3	4·6	17	3·7
Small intestine	2	—	..	..	..
Large intestine exc. rectum	49	1	2·0	13	2·9
Rectum	36	1	2·8	10	2·2
Extra-hepatic ducts	14	—	..	..	..
Liver (secondary)	3	—	..	..	..
Pancreas	13	—	..	..	..
Peritoneum	5	—	..	..	..
Larynx	2	—	..	..	..
Trachea, bronchus and lung	19	2	10·0	20	4·3
Mediastinum	2	—	..	..	..
Breast	77	—	..	..	..
Cervix uteri	20	—	..	..	..
Corpus uteri	5	—	..	..	..
Uterus, unspecified	14	—	..	..	..
Ovary and fallopian tube	21	—	..	..	..
Unspecified fem. gen. organs	2	—	..	..	..
Kidney	4	—	..	..	..
Bladder and urin. organs	11	—	..	..	..
Malignant melanoma of skin	1	—	..	..	..
Other malignant neoplasm of skin	3	—	..	..	..
Eye	1	—	..	..	..
Brain and other parts of N.S.	4	—	..	..	..
Thyroid gland	2	—	..	..	..
Bone (including jaw bone)	3	—	..	..	..
Secondary and unspec. malig. neoplasm of lymph nodes	1	—	..	..	..
Other and unspecified sites	16	—	..	..	..
Lymphosarcoma and reticulosarcoma	2	—	..	..	..
Hodgkin's disease	3	—	..	..	..
Lymphatic leukaemia	1	—	..	..	..
Myeloid leukaemia	2	—	..	..	..
Acute leukaemia, type unspecified	4	—	..	..	..
	417	7	..	..	..

N.B.—To reduce the complexity of presentation the detailed site distribution of all cancer deaths in the two groups of husbands is omitted from this Table. It is available on request.

This conclusion is not invalidated by the fact that we cannot draw up a hard and fast list of habits that are or are not always either individual or common to husband and wife.

The above remarks are likely to be true even if we assume that a complex of factors in heredity and environment (both inside and outside the home) is needed to set the stage necessary for a particular growth to occur.

Can our negative result be interpreted as meaning that, in future endemiological investigations based on questionnaires, we can exclude inquiry into domestic habits usually common to members of the same household? It might; but because

this could lead to neglect of a large field of inquiry it would be safer to say " The negative result suggests that an investigation of domestic habits (in relation to cancer aetiology) would be more likely to be profitable if directed towards habits usually peculiar to individuals in a household than towards those common to most members of the household ". Nevertheless, although this wide, negative finding might guide future investigations, it should not limit their field, lest some important fact should, by neglect, fail to be revealed.

CONCLUSION

There is no evidence, from the present investigation, to confirm the hypothesis that domestic factors or habits common to husband and wife are carcinogenic.

The results seem to suggest that it would be less profitable to investigate the possible carcinogenic influence of common domestic factors than other, usually unshared, factors. However, this conclusion should be applied with caution to future plans for endemiological enquiries lest a conceivably important field for further investigation be neglected.

SUMMARY

This investigation attempts to answer the question " How important are shared domestic environmental factors in the aetiology of cancers?". To eliminate family genetic factors a study was made of the causes of death of husbands and wives.

The material consisted of 417 widows who died from cancer (" cancer widows ") and their husbands, the causes of all the deaths being known. A carefully matched control group of 455 widows who did not die from cancer (non-cancer widows) together with their husbands, the causes of whose deaths were also known, was used for comparison. The cancer widows and control widows were closely comparable in respect of age, social class, place of residence, date of death, etc.

It was found that 20 per cent of the husbands of cancer widows died from cancer. The percentage of husbands of non-cancer widows who died from cancer was 20·6 per cent.

It is concluded that, as cancer in the husbands of women who died from cancer was no more frequent than cancer in the husbands of women who did not die from cancer, then it is unlikely that shared domestic environmental factors are important carcinogenic agents for the time, places and people in this study.

An appendix describes the observed percentage of cancers of identical sites in cancer widows and their husbands and compares these with the findings in the group of non-cancer-widows and their husbands.

NOTE

When this paper was written the literature was searched under the heading " Cancer in Husbands and Wives " without result. Because the author happened to come across a reference to Ciocco's work the literature was re-searched under the heading " Mortality in Husbands and Wives ". This revealed Ciocco's (1940, 1941, 1942) papers. Using an approach similar but not identical to the method described in the present paper Ciocco found an excess of cancer above expectation in the spouses of cancer subjects, i.e. his results are the reverse of our own.

Evelyn A. Potter and Mildred R. Tully, deliberately following Ciocco's methods as closely as they could in Massachusetts, failed to confirm his results. Their findings, then, are compatible with our own, though they used a somewhat different method.

Obviously from the above conflicting results further investigations are required and until these have been carried out, generalisations about the presence or absence of association of cancers in husbands and wives are unjustifiable.

Thanks are due to the following Medical Officers of Health for allowing access to their records for this research :

Dr. E. J. MacIntyre	(Banstead U.D.C.)
Dr. W. Alastair Glen	(Eastleigh M.B.)
Dr. M. I. Adams	(Fulham M.B.)
Dr. Percival W. Pritchard	(Gosport M.B.)
Dr. W. D. Swinney	(Merton and Morden U.D.C.)
Dr. Robt. A. Good	(City of Winchester).

My special thanks are due to Miss Joyce Strudwick and Miss Rosemary Axe, B.A. who carried out most of the investigation. Thanks for co-operation are due to Miss V. P. Farrow and her staff at the South West London Mass X-ray Service, especially to Mrs. V. Collins, Mrs. M. Levett, Mrs. W. E. Smith, Miss A. Taylor, Mrs. V. Thomas and Miss K. E. Janvrin Tims.

I am also grateful to the Librarian of the Royal Society of Medicine for searching the literature.

I am required to state that this investigation formed part of a successful entry for the South West Metropolitan Regional Hospital Board Research Prize Competition 1958.

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APPENDIX

Table IX shows the type occurrence of the 417 cancers occurring in the cancer widows (col. 1). Column 2 of this table shows the number of deaths from cancer of the same site occurring among the husbands of women dying from cancer of that particular site.

Of 65 women who died from carcinoma of the stomach, 15 (23 per cent) had husbands who died from cancer of which 3 died from cancer of the stomach, i.e. 4·6 per cent, whereas of the 455 women not dying from cancer, 17 had husbands die from stomach cancer (3·7 per cent) and of the 352 women who died from cancer of other sites the husbands of 10 died from cancer of the stomach (2·8 per cent).

Of 49 women who died from carcinoma of the colon, 8 (16·4 per cent had husbands who died from cancer of which 1 died from cancer of the colon, i.e. 2 per cent whereas of the 455 women not dying from cancer 13 had husbands die from carcinoma of the colon (2·9 per cent) and of the 368 women who died from cancer of other sites the husbands of 7 died from cancer of the colon (1·9 per cent).

Of 36 women who died from carcinoma of the rectum, 8 (22 per cent) had husbands who died from cancer of which 1 died from cancer of the rectum, i.e. 2·8 per cent whereas of the 455 women not dying from cancer 10 had husbands die from cancer of the rectum (2·2 per cent), and of the 381 women who died from cancer of other sites the husbands of 7 died from cancer of the rectum (1·8 per cent).

Of 19 women who died from lung cancer 5 (26·3 per cent) had husbands also die from cancer of which 2 died from lung cancer, i.e. 10 per cent, whereas of 455 women who died from causes other than cancer, 20 had husbands die from lung cancer (4·3 per cent), and of the 398 women who died from cancer of other sites the husbands of 17 died from lung cancer (4·4 per cent).

We have already described the absence of unexpected association of the same cancer in husband and wife in respect of the following sites :— stomach, colon, rectum and lung. Looking down the list of remaining sites no example is seen of the same growth occurring in husband and wife among the less common growths.

Examination of the series for evidence of associated causes of death in husbands and wives other than malignancy was undertaken but will not be described here, the number of diseases being so large in proportion to the number of deaths available that no reliable conclusions are possible. For the same reason it was not possible to make a reliable investigation into the possibility of an association between the occurrence of a particular site of cancer in the wife and some other specific non-malignant disease in the husband.

THE INCREASE IN LUNG CANCER MORTALITY IN CANADA

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Received for publication September 7, 1959

In a previous paper (Phillips, 1954) it was shown that in Canada between 1931 and 1952 deaths from lung cancer had increased approximately six times among males and had more than doubled among females. The experience in numerous other countries in lung cancer mortality has been reported (Clemmesen, Nielson and Jensen, 1953; Dorn, 1953; Stocks, 1952) and equal or greater increases shown. Such studies are based, generally, upon official mortality statistics. However, in a disease which, for its correct identification, requires medical skills and facilities that are only recently coming into widespread use, the assessment of increases in lung cancer mortality requires a careful study of the relative accuracy of death certificates and special consideration for the predominant pulmonary diseases of the period under review.

Greenwood (1948) has stated that a change of fashion in death certification due to increasing knowledge may affect comparability and more recently Gilliam (1955) has written that, in lung cancer, although a large majority of informed opinion accepts the belief that the increase being experienced in many countries is real, considerable divergence of opinion remains with regard to its magnitude. Herdan (1958) has suggested that, when examining striking changes in the incidence of non-epidemic disease one must be mindful of the possibility of a change in diagnosis. In order to study such comments as they apply to mortality data in Canada a review has been made of deaths attributed to the major diseases of the respiratory system for the period 1931–1956. The sexes have been analyzed separately between the ages of 35 and 84 years. All mortality rates have been standardized on the 1951 census population of Canada in order to permit direct comparisons. The following causes of death with the International List Number were selected for study :—

I. Malignant neoplasms of trachea, bronchus and lung not specified as secondary—
- 47 (a), (b), (c), (d) . . . . . 4th Revision
- 47 (a), (b), (c), (d), (e), (f) . . . . . 5th Revision
- 162, 163 . . . . . 6th Revision

II. Tuberculosis of the respiratory system—
- 23 . . . . . 4th Revision
- 13 . . . . . 5th Revision
- 0001 to 0008 . . . . . 6th Revision

III. Diseases of the respiratory system—
- 104 to 114 . . . . . 4th Revision
- 104 to 114 . . . . . 5th Revision
- A 87 to 97 (Intermediate List) . . . . . 6th Revision

III. (a). Bronchopneumonia—
- 107 . . . . . 4th Revision
- 107 . . . . . 5th Revision
- 491 . . . . . 6th Revision

III. (b). Lobar pneumonia—
108 . . . . . 4th Revision
108 . . . . . 5th Revision
490 . . . . . 6th Revision

III. (c). Pneumonia (unspecified)—
109 . . . . . 4th Revision
109 . . . . . 5th Revision
492, 493 . . . . . 6th Revision

The standardized death rates are shown for males in Table I and Figure 1 and for females in Table II and Fig. 2. From the tables it will be noted that lung cancer mortality in males has increased from 6·9 per 100,000 population in 1931 to 56·9 in 1956, an increase of 8·2 times, while the female rates have increased approximately 2·4 times. On the other hand the tuberculosis rates have decreased from 80·8 per 100,000 to 19·2 for males and from 64·2 to 8·7 for females. All other respiratory diseases have decreased for males from 138·2 per 100,000 to 83·4 and for females from 125·7 to 44·0 per 100,000.

TABLE I.—*Standardized Death Rates Per* 100,000 *Population for Selected Causes Among Males* 35 *to* 84 *Years* (1931–1956)

Years	Lung cancer	T.B.	All respiratory	Pneumonia		
				Broncho	Lobar	Unspecified
1931	6·9	80·8	138·2	34·4	42·0	22·3
1932	7·4	75·5	138·0	36·9	45·7	22·6
1933	8·8	72·1	135·8	36·9	42·0	18·5
1934	8·6	72·2	135·0	35·7	45·0	15·0
1935	10·7	73·9	141·8	40·2	53·0	15·1
1936	10·9	72·9	144·6	44·3	52·8	14·7
1937	12·1	70·6	148·0	44·3	52·6	13·5
1938	12·4	65·1	140·6	40·2	54·0	13·0
1939	15·5	64·7	122·0	41·7	36·7	11·6
1940	17·3	64·4	114·5	36·7	32·9	9·9
1941	17·6	69·6	93·0	29·4	24·1	11·1
1942	16·9	66·1	90·4	30·3	25·2	10·7
1943	18·7	69·2	101·8	31·2	30·7	11·9
1944	18·5	63·4	91·8	28·6	26·2	11·7
1945	21·3	61·6	81·8	24·8	22·8	10·7
1946	24·4	61·2	84·8	27·5	19·6	10·7
1947	28·4	64·9	78·7	27·5	18·5	9·1
1948	29·5	57·6	78·5	27·7	18·8	9·0
1949	32·9	52·4	80·3	26·3	17·5	10·6
1950	36·9	46·4	73·3	20·2	12·2	8·0
1951	38·7	43·9	96·0	24·3	10·9	9·8
1952	42·0	33·4	68·4	22·0	8·1	7·1
1953	47·0	25·4	78·0	21·8	10·3	8·9
1954	50·1	24·0	66·4	20·7	8·3	8·2
1955	53·6	20·6	74·9	25·7	7·5	8·2
1956	56·9	19·2	83·4	27·2	10·3	7·9

The comments of Greenwood and Herdan regarding a change of fashion or a change of diagnosis in lung cancer death certification may be investigated by a study of the assumption that there has been no real increase in lung cancer mortality in Canada and that increases in death rates are due entirely to improvements in death certification. If this be true then following the proposal of Gilliam (1955), the number of deaths presumably resulting from cancer of the lung in 1931 may be calculated by applying the 1956 age-specific rates for each sex to

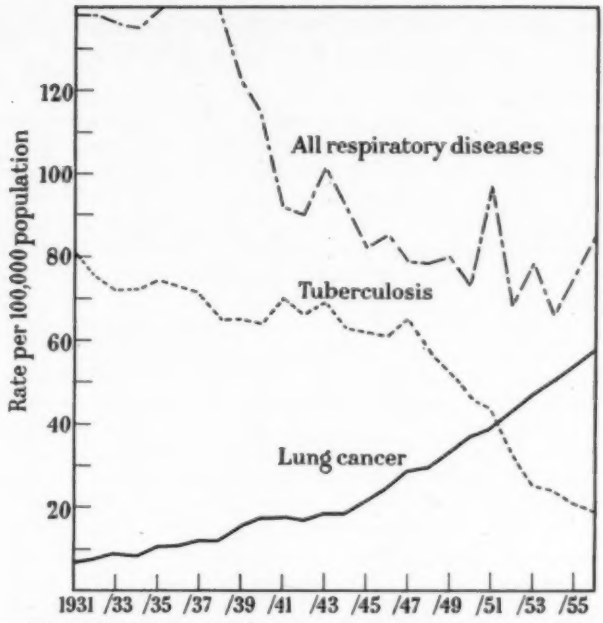

FIG. 1.—Standardized death rates for selected causes among males 35 to 84 years.

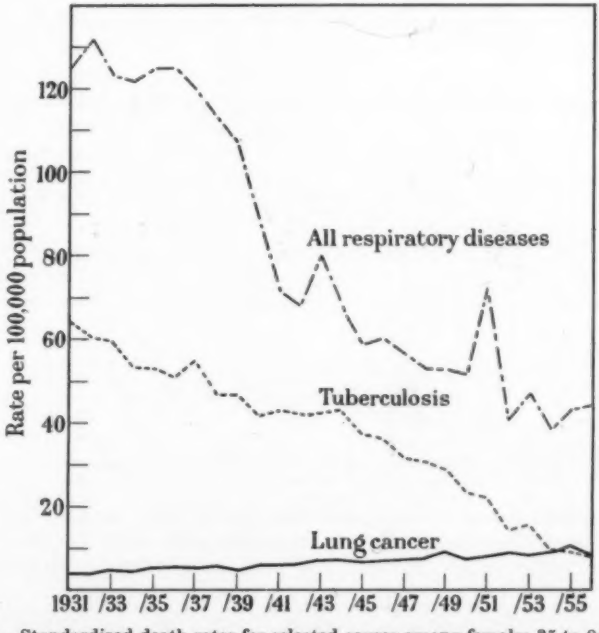

FIG. 2.—Standardized death rates for selected causes among females 35 to 84 years.

TABLE II.—*Standardized Death Rates Per* 100,000 *Population for Selected Causes Among Females* 35 *to* 84 *Years* (1931–1956)

Years	Lung cancer	T.B.	All respiratory	Pneumonia		
				Broncho	Lobar	Unspecified
1931	3·8	64·2	125·7	38·4	33·6	22·1
1932	3·9	60·5	131·6	38·0	41·5	24·3
1933	5·0	59·6	123·8	39·9	35·5	18·2
1934	4·8	53·5	121·9	37·3	36·8	16·9
1935	5·4	52·2	124·8	41·8	41·1	15·0
1936	6·1	51·3	124·9	38·4	39·6	14·1
1937	5·6	54·6	120·0	42·5	41·6	12·0
1938	5·9	47·2	112·7	40·3	37·0	12·0
1939	5·2	46·7	107·3	38·6	31·8	11·1
1940	6·2	42·3	89·4	34·1	21·9	9·1
1941	6·2	43·2	71·6	23·3	18·5	10·1
1942	6·1	41·9	68·0	24·1	15·8	9·6
1943	7·4	42·5	79·7	27·6	19·6	10·6
1944	7·5	43·0	68·1	23·6	18·5	9·6
1945	7·0	37·4	58·9	21·0	14·2	7·7
1946	7·0	36·5	60·4	20·8	13·4	10·0
1947	7·4	31·8	57·0	22·5	12·0	7·8
1948	7·9	31·1	53·2	20·5	11·6	5·8
1949	9·5	29·3	52·8	20·0	10·0	8·3
1950	7·5	23·6	52·1	16·8	7·2	7·7
1951	8·2	22·5	72·4	19·2	5·7	7·0
1952	9·2	14·6	41·0	14·0	4·7	5·8
1953	8·5	11·1	47·5	16·5	4·8	5·2
1954	8·9	10·0	38·5	14·4	4·5	5·4
1955	10·8	9·8	43·0	16·2	4·3	5·4
1956	9·2	8·7	44·0	16·8	4·3	7·0

the appropriate 1931 populations. The results so obtained, less the deaths actually recorded in 1931 give the deaths from cancer of the lung that on this assumption were, in 1931, incorrectly attributed to some other disease. These are given for males and females in Table III.

TABLE III.—*Additional Deaths from Lung Cancer Needed in* 1931* *Shown as Percentages of Deaths from all Respiratory Diseases Including Tuberculosis*

Age Group	Additional lung cancer deaths needed in 1931		Additional lung cancer deaths as proportion of deaths from all respiratory diseases	
	Male	Female	Male (%)	Female (%)
35–39	6	2	1·6	5·3
40–44	20	0	4·8	0·0
45–49	40	5	9·2	2·0
50–54	95	6	22·0	2·9
55–59	140	14	39·0	7·0
60–64	158	6	46·6	2·6
65–69	165	13	41·5	4·3
70–74	123	16	30·1	4·1
75–79	59	14	17·0	3·7
80–84	31	5	10·8	1·5

* In order that the 1931 and 1956 lung cancer mortality rates be equal.

It would seem reasonable to assume that deaths from cancer of the lung might be confused more frequently with tuberculosis and other respiratory diseases, than with any other cause. If there had been no increase in lung cancer deaths

between 1931 and 1956 and the additional deaths needed in 1931, shown in Table III, had been erroneously diagnosed as some other respiratory disease including tuberculosis, then these needed deaths divided by the recorded deaths for respiratory diseases including tuberculosis give the percentages of error in diagnosis of these diseases necessary in each age and sex group. These percentages are given in Table III and show that for the age-specific lung cancer rates to remain the same the diagnostic error in all respiratory diseases including tuberculosis would have ranged from 1·6 per cent, for males aged 35 to 39 years to 46·6 per cent for those aged 60 to 64 years. In addition different percentages of error would have been required for females. In order that the basic assumption be upheld viz. that increases in lung cancer death rates are due entirely to improvements in death certification, it is necessary to show that errors in diagnosing lung cancer as other respiratory diseases occurred more frequently in males than females and more frequently in the older age groups. Since there is not a consistent increase of diagnostic error with age the basic assumption must be rejected and the data interpreted to indicate that an increase in cancer of the lung has in fact occurred.

It is difficult to determine precisely how well the recorded mortality measures the magnitude of this increase. Numerous examples have been presented, especially in earlier years, showing the erroneous diagnosis of lung cancer as tuberculosis, and post-mortem studies such as reported by Waller and Grimstvedt (1958) indicate that a substantial diagnostic error may occur in carcinoma of the lung. More recently the use of antibiotics has permitted a number of patients to survive until the correct diagnosis was made whereas they would have succumbed formerly to pneumonia or some other respiratory cause before the discovery of the underlying cancer of the lung. Unfortunately there is no way to estimate directly to what extent such circumstances have affected the recorded increase in lung cancer. This may be done indirectly by measuring the effect of a selected percentage of error in the diagnoses of respiratory diseases on the mortality trend of lung cancer. Assuming a 2 per cent and a 5 per cent error of diagnosis the deaths resulting from the assumptions are deducted from the recorded number and transferred to cancer of the lung.

Table IV shows the results of these calculations and it will be noted that, whereas in males the recorded lung cancer mortality rates increased 8·2 times, an error of 5 per cent reduces this to 3·6 times. In females the increase is reduced from 2·4 times, to no increase. If a 2 per cent error in diagnosis is assumed the increase in lung cancer mortality for males is 5·3 times and 1·4 times for females. This procedure assumes that the error factor is constant throughout the period under review whereas it would seem more reasonable to assume that errors in

TABLE IV.—*Effect of a Five Per cent. Error in the Diagnosis of Respiratory Diseases on the Mortality From Lung Cancer*

Sex	Standardized lung cancer death rate (1931)	Standardized lung cancer death rate (1956)	Number of times increase	Standardized lung cancer death rate including 5% error (1931)	Standardized lung cancer death rate including 5% error (1956)	Number of times increase
Male	6·9	56·9	8·2	17·6	64·0	3·6
Female	3·8	9·2	2·4	13·4	11·9	None

diagnosis have decreased in recent years. If the 5 per cent error factor is applied only to 1931 deaths and the 1956 deaths assumed to contain no error the increase in lung cancer mortality in males would be reduced further, to 3·2 times.

It is to be noted also that an assumption of a constant error in the diagnosis of all respiratory diseases including tuberculosis results in a decrease in the error in diagnosis of lung cancer. Referring to Table IV the calculated lung cancer rate in 1931, assuming an error of 5 per cent in respiratory disease diagnoses, is 17·6 for males and 13·4 for females while the recorded rates are 6·9 and 3·8. Thus on this assumption 39·2 per cent (6·9/17·6) of the male lung cancer and 28·4 per cent (3·8/13·4) of the female were correctly diagnosed in 1931. In 1956, however, the corresponding proportions are 88·9 per cent (56·9/64·0) for males and 77·3 per cent (9·2/11·9) for females. It seems evident therefore that, as deaths from all respiratory diseases decline there is a decreasing possibility that errors in diagnosing them could contribute substantially to the death rate from lung cancer.

Whether the two per cent or the five per cent error in diagnosis is considered acceptable it seems reasonable to assume that errors in the diagnosis of lung cancer occur and may have occurred more frequently twenty-five years ago. On this assumption the increases, since 1931, in the recorded lung cancer death rates in Canada for both sexes are probably higher than in actual fact.

SUMMARY

A study has been made of the experience in Canada in deaths from lung cancer and other major respiratory diseases. The period reviewed was 1931 to 1956. The standardized death rates indicate that lung cancer has increased approximately eight times among males and doubled among females. Significant declines for each sex were found in deaths from tuberculosis and from all other respiratory diseases. The assumption that all of the increase in mortality attributed to lung cancer since 1931 could be accounted for by erroneous death certification to other respiratory diseases including tuberculosis cannot be substantiated without unreasonable assumptions of age and sex differences in diagnostic error. It is shown also that a substantial proportion of the recorded increase can be accounted for theoretically by a diagnostic error of five per cent. Thus if five per cent. of the deaths attributed to respiratory diseases including tuberculosis were actually due to lung cancer, the recorded increase in males since 1931 in Canada would be 3·6 times instead of 8·2 times and in females no increase would be evident instead of one of 2·4 times. The increase would be reduced somewhat further on the assumption of greater diagnostic error in the earlier than in the later years under review.

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MALIGNANT DISEASE OF THE GASTRO-INTESTINAL TRACT IN SINGAPORE

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Received for publication October 1, 1959

SINGAPORE is singularly suitable, as is neighbouring Malaya, for the study of what has become known as Geographical Pathology. The presence of a mixed population of some one and a half millions, 75 per cent Chinese, 14 per cent Malay, and 9 per cent Indian and Pakistani, within an island of some 217 square miles, makes for an epidemiological unit of convenient size, all the more so as there is but one general hospital. Since the island lies one degree north of the equator, and one hundred and four degrees east of Greenwich, the climate is hot (average 85° F.), moist (average relative humidity 83 per cent), and equable. There are no seasons.

METHODS : MATERIAL : SOURCES OF ERROR

At the mortuary of the General Hospital, Singapore, post-mortem examinations are carried out on persons who have died in the hospital, and on cases falling within the aegis of H.M. Coroner. In addition, a morbid anatomist from this department visits the Kandang Kerbau Maternity Hospital : post-mortem records are thus available from these three sources. A few post-mortem examinations carried out at a tuberculosis and a military hospital are not included. In short, records of almost all autopsies carried out in Singapore are available to this department.

All the post-mortem records of cases of malignant disease were scrutinised by the author and the data considered relevant were entered on a specially prepared pro forma for subsequent analysis.

This study covers the years 1948–58 inclusive, when 22,997 post-mortem examinations were carried out. In this material there were 304 deaths from malignant disease of the gastro-intestinal tract, i.e., 1·32 per cent. If the 11,525 children under the age of 11 years be excluded then the incidence is 2·65 per cent.

The age and sex incidence, by race, for the post-mortem population for the years 1950–58 inclusive is given in Table I, and the mean age and sex incidence, by race, for the entire population of Singapore for the period mid-1947 to mid-1957 is given in Table II. As most values have been approximated to the nearest round number some of the cross-additions may be apparently incorrect.

The sex disparity, once very great, in the population has been almost corrected since the cessation of unrestricted immigration. However, it will be noted that there is still an undue preponderance of Indian males in the third and subsequent decades.

Unfortunately, it has proved impossible to compile much of the data in this paper for the whole of the period covered by the post-mortem survey, and only such figures as were available have been presented.

TABLE I.—*The Age and Sex Distribution of the Singapore Post-Mortem Population, 1950–58 Inclusive*

Age	Chinese		Malay		Indian		Others		Total		Grand total	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Number	Per cent
0–10	5,150	3,980	75	43	165	117	32	20	5,422	4,160	9,582	49·6
11–20	555	274	45	15	32	25	14	0	646	314	960	5·0
21–30	685	353	73	27	160	36	58	13	976	429	1,405	7·3
31–40	844	366	59	14	295	50	56	9	1,254	439	1,693	8·8
41–50	1,382	404	52	12	326	23	55	14	1,815	453	2,268	11·7
51–60	1,365	278	45	10	256	22	58	6	1,724	316	2,040	10·6
61–70	713	191	6	3	81	13	25	5	825	212	1,037	5·4
71+	191	95	10	7	15	4	9	5	225	111	336	1·7
Totals	10,885	5,941	365	131	1,330	290	307	72	12,887	6,434		
	16,826 (87·0%)		496 (2·6%)		1,620 (8·4%)		379 (2·0%)		19,321		19,321	100·1

TABLE II.—*Mean Age and Sex Distribution of the Singapore Population from Mid-1947 to Mid 1957*

Age	Chinese		Malaysians		Indians		Others		Total		Grand total	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Number	Per cent
0–9	143,940	133,398	25,478	25,707	11,187	11,004	3,718	3,631	184,323	173,739	358,061	30·0
10–19	97,985	89,156	14,149	14,754	7,266	5,365	2,020	2,234	121,419	111,509	232,927	19·5
20–29	69,194	66,466	18,222	14,262	15,518	5,426	2,982	2,702	105,917	88,855	194,772	16·3
30–39	59,800	54,546	12,477	8,780	17,966	3,226	3,074	2,562	93,317	69,113	162,431	13·6
40–49	53,054	45,015	7,103	5,063	10,934	1,562	2,160	1,561	73,250	53,202	126,453	10·6
50–59	32,162	28,270	3,725	2,580	4,597	703	1,193	935	41,677	32,489	74,166	6·2
60–69	12,388	15,074	1,436	1,254	1,101	272	435	453	15,360	17,054	32,414	2·7
70+	3,209	6,636	635	739	273	113	149	237	4,266	7,726	11,992	1·0
Totals	471,732	438,561	83,225	73,139	68,842	27,671	15,731	14,315	639,529	553,687		
	910,293 (76·3%)		156,364 (13·1%)		96,513 (8·1%)		30,046 (2·5%)		1,193,216		1,193,216	99·9

It will be seen that there is considerable disparity between the post-mortem rates for the various races, the relative racial proportions for the population as a whole being Chinese 76·3 per cent, Malay 13·1 per cent, Indians 8·1 per cent, and Others 2·5 per cent, while the corresponding figures for post-mortems are 87·0, 2·6, 8·4 and 2·0 per cent.

Hence, unless the disease process under study is likely to cause sudden death, when an autopsy would be carried out irrespective of race at the mandate of H.M. Coroner, any post-mortem series will be quite unrepresentative racially, as for religious and other reasons Malays virtually never give consent for autopsy. Thus great care must be taken to ensure that the figures are comparable before concluding that any given neoplasm or disease process is rare or common in a particular racial group. Further, the requirement for autopsy in cases of sudden death in a racial group which is averse to morbid examination will give a false impression of the importance as a cause of death of, say, coronary heart disease.

The age structure of the Singapore population tends to give a falsely low impression of the relative incidence of malignant disease, just over half the population being under 21 years of age (1957). Further, the rate of natural increase is such that by 1962 half the population may well be under 15 years.

Over the years 1906–10, when the mean population was about 250,000 the cancer mortality rate per 100,000 population was 12·6 (Hoffman, 1916). For the period 1926–31, when the population was approximately double, the incidence was 40·6 (Hoffman, 1935). In 1957 the overall incidence was 53·2, and for the preceding five years the mean rate was 51·9. This gradual rise and maintenance of the rate of incidence at a time of explosive natural increase suggests either that the incidence of cancer as a whole is rising, or that the public is making better use of the improved medical facilities.

As post-mortem figures of incidence, both absolute and relative, are usually held to be biased, it was felt that no fair comment could be made unless hospital admissions, hospital deaths and the returns of the Registrar-General, Singapore, gave roughly comparable figures. That they do so tally to a remarkable degree

TABLE III.—*Comparison of the Number and Relative Incidence of Hospital Admissions, Hospital Deaths, Post-mortems, and Deaths recorded by the Registrar-General, Singapore, for Malignant Tumours of the Gastro-Intestinal Tract, 1954–58 Inclusive*

International List No.		Hospital admissions		Hospital deaths		Post-mortems		Registrar-General	
		Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
150	Oesophagus	485	27	172	34	48	32	299	24
151	Stomach	856	48	253	50	87	58	712	58
152	Small gut	12	1	1	—	2	1	2	—
153	Large gut	179	10	43	8	7	5	112	9
154	Rectum	241	14	39	8	6	4	109	9

is shown in Table III. As the system of classification of the returns of both the hospital and the Registrar-General has altered several times in the past ten years it was not possible to complete this table for the period covered by the post-mortem survey.

The figures of the Registrar-General, Singapore, are probably the most accurate in South-East Asia as 55 per cent of deaths are certified by doctors, 12·5 per cent by H.M. Coroner, usually following a post-mortem examination, and 32·5 per cent by Police Officers and Hospital Assistants (1957). In neighbouring Malaya, in 1956, 80·7 per cent of deaths were not certified by a medical practitioner. The majority of deaths certified by unqualified persons are ascribed to "sawan" (convulsions) or "demum" (fever), (Griffith, 1958). Neoplasia is rarely, if ever, mentioned.

Neoplasms of the gastro-intestinal tract form a high proportion of all malignant tumours. In Table IV the incidence of these tumours, as recorded by the Registrar-General, Singapore, is given, and expressed as a percentage of the 3,777 malignant neoplasms recorded in the same period.

TABLE IV.—*Incidence of Gastro-Intestinal Tract Neoplasms Recorded by the Registrar-General, Singapore, 1952–57 Inclusive*

Site	Number	Per cent	Percentage of all malignant neoplasms, i.e. of 3777
Oesophagus	283	22·0	7·5
Stomach	783	61·0	20·7
Small intestine	6	0·5	0·2
Colon	109	8·5	2·9
Rectum	103	8·0	2·7
Total	1284	100·0	34·0

The oesophagus

There were 84 cases of oesophageal carcinoma, 73 males (87 per cent) and 11 females. The mean age at death for the 11 females, who were all Chinese, was 56·1 ± 13·4 years, and for the 69 Chinese males 53·9 ± 9·4 years, 2 Indian Hindus, 1 Indian Muslim and 1 Malay brought the male total to 73.

The peak incidence for males was in the decades 40–49 and 50–59, while for the females it was in the decades 50–59 and 60 –69.

Ochsner and de Bakey (1941) who collected 8572 cases of oesophageal carcinoma from the medical literature found that 20 per cent were situated in the upper-third, 37 per cent in the middle third, and 43 per cent in the lower third. Comparable figures for this series are 6 per cent, 50 per cent and 40 per cent. The site was not stated in the remaining 4 per cent.

The majority of the carcinomata were described as being about 10 cm. in diameter: obstruction was noted in 72 per cent.

Fistulae were common, occurring in 40 per cent of upper third, 38 per cent of middle third, and 10 per cent of lower third carcinomata. Those in the upper third communicated with the trachea; those in the middle third with the lung direct, the bronchi, the trachea and the pericardium in that order; those of the lower third with the lung direct and the pericardium.

Not unexpectedly, abscess formation was common. Fifteen per cent of unoperated cases showed a concomitant lung abscess, 5 per cent a mediastinal abscess and 2 per cent a brain abscess.

Metastatic spread was extremely common. The mediastinal glands were grossly involved in 45 per cent of cases, the abdominal glands in 18 per cent and the cervical glands in 11 per cent. There was spread to local tissues in 30 per cent, to the lung in 13 per cent, to the liver and brain in 10 per cent of cases. These figures are in rough agreement with those of Dormanns (1939) who analysed the sites of metastasis in 824 cases of oesophageal carcinoma.

Oesophageal carcinomata seem to be much commoner in Singapore than in the Occident, forming 27·6 per cent of this series, and about 8 per cent of all neoplastic deaths (Table IV).

Ackerman and Regato (1954) state that in the U.S.A. approximately 2 per cent of all deaths from carcinoma are oesophageal.

Oesophageal growths have long been known to be very common in China. Kwan (1937) found about half of gastro-intestinal growths were in the oesophagus, and a similar high incidence in the Chinese of Java has been noted (Kouwenaar, 1950).

Marsden (1958), in an analysis of biopsy material in Malaya, found carcinoma of the oesophagus in 19 per cent, 25 per cent and 17 per cent of gastro-intestinal tumours in Malays, Chinese, and Indians respectively.

Much stress has been laid on the consumption of hot food as an aetiological factor and, as the Chinese male was reputed to be the first in the family to eat from the dish, the male preponderance was held to be accounted for. This, as Marsden (1958) comments, is certainly not true for either Malaya or Singapore, and he suggests that the drinking of spirits is a much more likely cause. The relatively high incidence of oesophageal cancer in Japan has also been attributed to alcohol (Irisawa, 1933) and Steiner (1956) discusses this relationship at length. Despite the frequency with which the Chinese female in Singapore attempts suicide by drinking caustic soda, no history of such an episode was recorded in the 11 females, although in other reports the association has been noted (Bigger and Vinson, 1950).

The stomach

There were 164 cases of carcinoma of the stomach. Of these 143 (87 per cent) were male, and 21 (13 per cent) female.

One hundred and forty-seven were of Chinese race. Of these 128 were male, and 19 female. The mean age at death for the males was 51·7 $\pm$ 10·5 years and for the females 45·9 $\pm$ 11·7 years.

The peak incidence was found to be in the decades 40–49 and 50–59 for the male (in all, 67 per cent), and the decades 30–39 and 50 –59 for the female (respectively 37 per cent and 32 per cent).

There were 13 cases in Indians, of whom 12 were male. The mean age at death for the males was 48·2 $\pm$ 11·5 years, the peak incidence being in the decade 50–59.

The sites of the tumours are given in Fig. 1. It will be noted that there is some sex disparity in the incidence of greater curve tumours. As the number of females is small, the significance of this finding is doubtful. Nevertheless an interesting point is raised which may repay further study.

Evans (1956) finds that approximately 65 per cent of carcinomata are in the prepyloric region, 20 per cent on the lesser curve, and 4 per cent on the greater,

the remaining 11 per cent being found at either cardia or fundus. Oppolzer (1938) summarising 837 cases of gastric carcinoma found 54 per cent to be prepyloric, 29 per cent on the lesser curve, 7 per cent at the cardia, 2 per cent on the greater curve and 7 per cent to involve the entire stomach. The Singapore figures are thus substantially similar.

Gross metastasis was very common, being present in almost every case. The commoner sites were the regional nodes in 54 per cent ; the liver, by blood spread, in 33 per cent and, by direct extension, in 10 per cent ; the omentum in 20 per cent ; the para-aortic nodes in 11 per cent ; the pancreas, lung, bone and brain each in 5 per cent. High as these figures are, they are considerably lower than

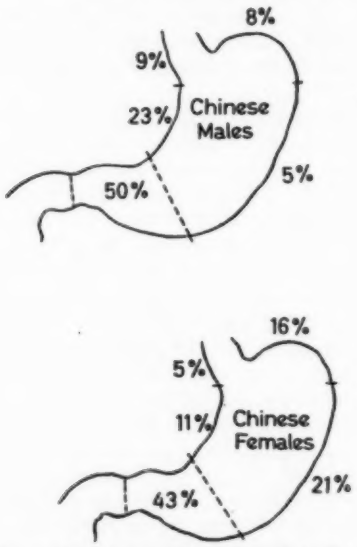

FIG. 1.—The site incidence of carcinoma of the stomach in Chinese males and females.

those of Stout (1943). Ovarian metastases were seen in 37 per cent of the females. Indeed, in one pregnant Chinese woman aged 40 years, the left ovary measured 28 by 16 by 10 cm., and weighed 2250 g., the right being approximately half this weight. Perforation of the malignant ulcer was observed in 25 cases (15 per cent), ascites in but 11 (7 per cent).

The returns of the Registrar-General, Singapore, show carcinoma of the stomach to be the commonest malignant tumour, accounting for one fifth of all deaths from malignant neoplasm. Marsden (1958) states that in Malaya the true incidence must be about 15 per cent. These figures are similar to those for the U.S.A. where, in 1948, 12·6 per cent of all cancer deaths were due to a gastric carcinoma.

Great stress has been laid on the frequency of gastric carcinoma in the Chinese as compared with the Malay, particularly in Java and Sumatra. In this series there were but two Malays (1·2 per cent) with gastric carcinomata. However, in Marsden's (1958) material the respective relative incidence for Malay, Chinese,

and Indians was 32 per cent, 35 per cent and 40 per cent of gastro-intestinal tumours, although on a population basis the relative incidence would be 5 per cent, 42 per cent and 53 per cent. It seems likely, in view of the strong aversion of the Malay to surgery, that the true relative racial incidence may in fact be much closer.

However, in a series of 1301 post-mortems on Chinese males, and 1189 on Javanese males, Kouwenaar (1951) found 120 and 59 malignant tumours respectively. Forty-six (38 per cent) of the tumours in Chinese were in the gut (oesophagus 24 per cent, stomach 67 per cent, colon 7 per cent, rectum 2 per cent), whereas there were only four in the Javanese. These figures, and others quoted by Kouwenaar (1955), rather suggest that there is a true racial difference in the incidence of these tumours. It will be noted that the figures for the Indonesian and the Singapore Chinese correspond closely.

In this series, if the incidence for male Chinese and male Indians + Pakistanis be compared with the mean male population for these races over the 11 years, then the relative incidence is virtually the same.

Small gut

There were eight tumours of the small gut representing 2·6 per cent of the intestinal malignancies. Four of these all in males, were lymphosarcomata ; the fifth in a female aged 38, was a sarcoma of doubtful origin ; the sixth, in a male aged 30 years was due to Hodgkin's disease ; the remaining two were adenocarcinomata occurring in a male aged 48 and a female aged 39 years. All were in persons of Chinese race.

Colon

In this series there were 35 colonic cancers 13 males (37 per cent) and 22 females (63 per cent). Of these, all but two were in Chinese.

The average age at death of the males was $52{\cdot}3 \pm 12{\cdot}8$ years and of the females, $56{\cdot}9 \pm 9{\cdot}3$ years. (A girl aged 9 years is excluded from this figure.)

The peak decade of incidence was, 50–59 for males and 60–69 for females.

Although the numbers involved are small there appears to be a sex difference in the site of the tumours as well. This is shown pictorially in Fig. 2. The sites are different to those found by Thompson (1957) in London.

Metastases were common. Fifty per cent of cases showed involvement of the regional nodes, 17 per cent extensive spread to local tissues, 11 per cent to the liver, and 6 per cent to the lungs. Carcinomatosis was seen in 6 per cent. Perforation was present in 46 per cent of cases : and a major degree of intestinal obstruction in a similar proportion.

The rarity of carcinoma of the colon in South East Asia has been commented on by Moore (1953) who spent five weeks in the region gathering the impressions of clinicians and pathologists.

Although in other reported series (Bacon, 1945) the sex incidence has been M/F 1·2 : 1, in Singapore the ratio is 0·6 : 1. This disparity is all the more striking if it be noted that for the other gastro-intestinal tract tumours the ratio is 6 : 1. Marsden (1958) found carcinoma of the colon to account for 15 per cent of gastro-intestinal carcinomata. He noted that there appeared to be no racial difference in incidence and that the M/F ratio was 1·5 : 1.

Rectum

In this series there were 13 rectal cancers, 9 in males (69 per cent) and 4 in females (31 per cent).

For the eight Chinese males the average age at death was 53·4 ± 13·2 years and for the four Chinese females 54·3 ± 11·2 years.

Metastases were common: to local tissues 46 per cent; to regional lymph nodes 62 per cent; to the liver 31 per cent and to the lung 23 per cent. Occlusion of the rectum was noted in 30 per cent. Most tumours were stated to be about 5 inches (12·7 cm.) from the anal margin.

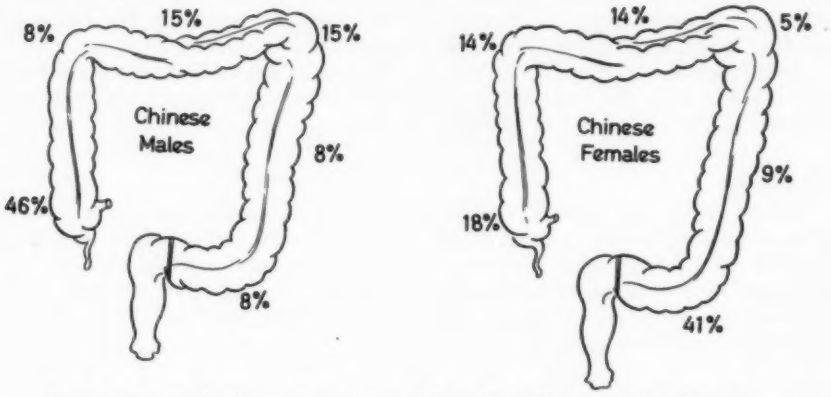

FIG. 2.—The site incidence of carcinoma of the colon in Chinese males and females.

DISCUSSION

It is of some interest to compare the post-war incidence of these tumours with pre-war figures.

Although most of the records of the Department of Pathology were destroyed during World War II, it has proved possible to trace a few charts and papers relating to a paper read at a scientific meeting by Dr. C. Subrahmanyam, lately Senior Pathologist, Singapore.

In 1937, 1939, 1940 and 1941, 383 post-mortems on persons having died with malignant disease were performed at Tan Tock Seng Hospital. (This institution was at that time a teaching general hospital, but during the period covered by this paper has been a tuberculosis sanitorium.) Of these, 145 (38 per cent) were gastro-intestinal, and of this 145, 43 per cent were oesophageal, 51 per cent gastric, and 6 per cent were found in the rectum and colon.

The late Dr. J. C. Tull, sometime Senior Pathologist, Singapore, provided comparative data for a paper by Bonne (1937) on the relative incidence of malignancy in the then Dutch East Indies. In a total of 128 cancers, 50 were gastro-intestinal. Of this 50, 30 per cent were oesophageal, 58 per cent were gastric, and 12 per cent were described as rectal.

It would thus appear that whatever may be causing the increased absolute

incidence in cancer, the relative proportions of the various gastro-intestinal tumours have remained, by and large, much the same.

In the U.S.A. Boles (1958) has noted a gradual decline in the incidence, both absolute and relative, of gastric carcinoma, with an increased incidence of tumours of the colon.

A comparison of the Singapore figures for gastro-intestinal neoplasms with those for England and Wales and the U.S.A. is given in Table V. The increased Singapore incidence of oesophageal, and to a lesser extent gastric neoplasms, and the diminished proportion of those in the colon and the rectum seems much too large to be fortuitous.

TABLE V.—*Comparison of the Relative Percentage Incidence of Gastro-Intestinal Tract Malignant Neoplasms, based on the Figures of the Registrar-General, Singapore, for* 1952–58 *inclusive, the Figures of the Registrar-General, England & Wales, for* 1940–42 (*Kennaway,* 1950) *and those of Boles,* (1958) *for the U.S.A. in* 1955

Site	Singapore	E. and W.	U.S.A.
Oesophagus	23·2	7·2	7·0
Stomach	59·5	41·9	35·0
Small gut	0·4	0·6	40·4
Large gut	8·7	30·9	
Rectum	8·2	19·3	17·5
Percentage all malignant neoplasms	33·8	47·5	40·0

Marsden (1958) eliminated the effect of the age structure of the Malayan population, which is very like that of Singapore, by plotting against the various age groups an index number derived from the ratio of cancer in each age group to the population in that age group each expressed as a percentage. Fig. 3 shows the age distribution of gastro-intestinal tract malignancies, corrected for population structure by this device. The graph follows Occidental figures fairly closely, although the small number of old people in the population has some effect on the width of the curve, the maximum incidence thus being in middle age.

Tempting though it may be to compare the Singapore figures with those reported from neighbouring territories in South-East Asia, such comparison is at best extremely crude. Cooray (1954) has pointed out the difficulties in coming to a reliable estimate of cancer deaths in Ceylon, even on the basis of post-mortem figures, as only 6 per cent of hospital deaths come to post-mortem. The figures of the Registrar (Ceylon) are also open to criticism as many deaths are certified by sanitary inspectors (Padley, not yet published). Biopsy figures for Ceylon are much more complete, but show what seems an unduly high proportion of rectal carcinomata, perhaps because such tumours are much more readily reached with the biopsy forceps. Three per cent of 1815 malignant biopsies were gastro-intestinal; oesophagus 18 per cent, stomach 13 per cent, small intestine 4 per cent, colon 24 per cent, and rectum 40 per cent. In South Vietnam a similar "cancerogramme" obtains (Joyeux and Nguyen-Van-Nguyen, 1953; Nguyen-Van-Ai, 1958). Of 3118 cancers 2·5 per cent took origin in the gut; oesophagus 2 per cent, stomach 24 per cent, small intestine 13 per cent, colon 16 per cent, and rectum 45 per cent. While such figures are a necessary preliminary in the investigation of the malignant epidemiology of a region, they may well differ considerably from the true incidence. Subrahmanyam (1953) records

Singapore biopsy figures for 1950 and 1951. In 595 malignant biopsies there were 54 from the gut; oesophagus 20 per cent, stomach 28 per cent, colon 28 per cent, and rectum 24 per cent. These values are at complete variance with the hospital, autopsy and Registrar-General's given in Table III.

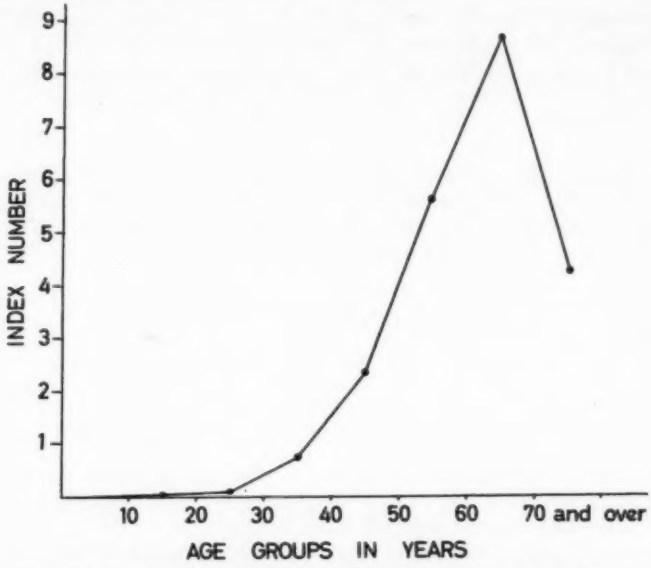

FIG. 3.—The age distribution of gastro-intestinal malignant neoplasms corrected for population structure.

$$\text{Index number} = \frac{\text{Per cent cancer in each age group.}}{\text{Per cent population in that age group.}}$$

SUMMARY

The morbid features of a post-mortem series of 304 gastro-intestinal malignant neoplasms seen in Singapore over the years 1948–58 inclusive are described.

The post-mortem incidence of these tumours is found to be: oesophagus 27·6 per cent; stomach 53·9 per cent; small intestine 2·6 per cent, colon 11·5 per cent; rectum 4·3 per cent. Comparison with hospital admission and death rates and with the figures of the Registrar-General, Singapore, for the same tumours, reveals a remarkable similarity.

Comparison of the figures of the Registrar-General, Singapore, with similar data from England and Wales, and the U.S.A. shows an increased frequency of oesophageal malignancies and a diminution of incidence of colonic and rectal tumours.

Attention is drawn to the population and racial structure of Singapore and some of the fallacies attendant upon comparison of incidence of disease between racial groups are noted.

The incidence of all malignancies has risen from 12·6 in 1906–10 to 51·9 per

100,000 population in 1952–6. No opinion can be given as to whether this rise is real or apparent.

Over the years it appears that the relative incidence of the various gastro-intestinal tract tumours in post-mortem material has remained fairly constant.

I wish to thank Professor R. Kirk for kind help and encouragement, my colleagues in the University and Government Departments of Pathology for access to their post-mortem notes, Mr. E. J. Phillips, Registrar-General, Singapore, for certain data, and Mr. Daniel Liu for assistance with the calculations.

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THE RELATIONSHIP BETWEEN AORTIC ATHEROSCLEROSIS AND CANCER

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Received for publication August 27, 1959

A REDUCED degree and frequency of aortic atherosclerosis have been reported in patients with cancer compared with non-cancer controls (Foldes, 1949; Wanscher, Clemmesen and Nielsen, 1951; Juhl, 1955; Creed, Baird and Fisher, 1955; Elkeles, 1956). These observations have been made both on autopsied groups (Wanscher, Clemmesen and Nielsen, 1951; Juhl, 1955; Creed, Baird and Fisher, 1955) and on groups of living patients (Foldes, 1949; Elkeles, 1956). However, since death from cancer frequently follows prolonged malnutrition and starvation, conditions favoring remission of atherosclerotic lesions, conclusions relative to this hypothesis are open to question when based on autopsy observations. On the other hand, Elkeles (1956) used roentgenologic diagnoses of calcification of the abdominal aorta to study the hypothesis in living patients. This seemed reasonable since it has been shown that there is a high correlation between roentgenological and pathological findings (Hyman and Epstein, 1954). Elkeles confirmed the hypothesis that there is a dissociation between cancer and aortic atherosclerosis in general and extended it to include the concept that this dissociation holds primarily for cancers which are influenced by genetic elements or are hormone dependent but not for those caused by exogenous carcinogenic agents. Unfortunately, the control groups utilized by Elkeles were drawn from general hospital populations and might well be selected with respect to the prevalence of aortic atherosclerosis.

Since these observations may be of importance as an indication of the influence of various metabolic and hormonal factors on cancer and atherosclerosis, we thought it would be of interest to try to confirm them on a population of patients admitted to the Roswell Park Memorial Institute, a cancer hospital. The advantage of this patient population is that, despite the fact that all patients admitted to the hospital have a suspect diagnosis of cancer, nearly half of the admissions are ultimately shown to be free of cancer. This latter group of patients serves as a " control " for comparison with the cancer group.

METHOD OF STUDY

As the study population, we selected 1462 consecutive admissions of white patients forty or more years of age during 1955. All patients had a posterior-anterior chest radiogram on admission; these were utilized for a diagnosis of aortic atherosclerosis. X-ray films on 1405 (96 per cent) of the study group were located. The radiologist (R. L) read these films with respect to presence of athero-

sclerosis, without the knowledge of the age or diagnosis of the patient. Information on diagnoses and other characteristics of interest were subsequently abstracted and coded directly from the hospital charts. The frequency of atherosclerosis among the cancer patients was then compared with a similar frequency in the "control" group.

To check on the validity of the radiological diagnosis of aortic atherosclerosis, a separate study was carried out utilizing autopsy material as well as admission radiograms. The pathologist (J. P.) graded 192 aortas for severity of atherosclerosis. These determinations were repeated after an interval of two months without knowledge of the first determinations. The results of this dual reading of degree of aortic atherosclerosis are shown in Table I. In 72 per cent of the dual readings both determinations were the same. In only 3 instances did first and second determinations differ by more than one grade.

TABLE I.—*Comparison of First and Second Pathological Determination of Degree of Aortic Atherosclerosis; Atherosclerosis Severity Graded 0–4*

First determination	Second determination					
	0	1	2	3	4	Total
0	—	—	—	1	1	2
1	—	17	12	—	—	29
2	—	1	29	18	—	48
3	—	1	5	63	5	74
4	—	—	—	10	29	39
Total	—	19	46	92	35	192

The radiologist (R. L.) then read the admission radiograms of these 192 patients without knowledge of the pathological diagnoses. The comparison of the radiological diagnosis of aortic calcification (atherosclerosis) and the first pathological diagnosis is shown in Table II. It is apparent that the radiological diagnosis was not particularly sensitive since a large number of patients with serious atherosclerosis were missed by the radiologist who depended on evidence of aortic calcification to make a diagnosis. However, the radiological diagnosis was highly specific since only cases with atherosclerosis of grade two or greater, were diagnosed by the radiologist. In view of this specificity we considered that the radiological diagnosis of aortic atherosclerosis, based on a determination of the presence of aortic calcification, could serve as an adequate index to test the hypothesis of a dissociation between cancer and atherosclerosis of the thoracic aorta.

TABLE II.—*Comparison of Radiological Determination of Calcification of the Thoracic Aorta and First Pathological Diagnosis of Aortic Atherosclerosis*

Radiological diagnosis	Graded pathological diagnosis									
	0		1		2		3		4	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Present	—	—	—	—	4	8·3	14	18·9	20	51·3
Absent	2	100·0	29	100·0	44	91·7	60	81·1	19	48·7
Total	2	100·0	29	100·0	48	100·0	74	100·0	39	100·0

RESULTS

Of the 1406 patients included in the study, 826 (59 per cent) ultimately were diagnosed as having cancer. Of the total sample 653 (46 per cent) were males and 753 (54 per cent), females. The age distributions of patients according to sex and presence or absence of cancer are given in Table III. Since there are differences between the age distributions of the males and females, both with and without cancer, it was necessary to take age into account in all comparisons between cancer patients and non-cancer patients; this was done by computing age-standardized percentages using the direct method of standardization.

TABLE III.—*Age Distribution According to Sex and Presence or Absence of Cancer Diagnosis*

Age	Cancer present		Cancer absent		Total	
	Number of individuals	Percentage distribution	Number of individuals	Percentage distribution	Number of individuals	Percentage distribution
			Males			
40–49	46	11·0	49	20·8	95	14·5
50–59	110	26·4	65	27·5	175	26·8
60–69	131	31·4	68	28·8	199	30·5
70 and over	130	31·1	54	22·9	184	28·2
Total	417	99·9	236	99·9	653	100·0
			Females			
40–49	94	23·0	123	35·8	217	28·8
50–59	96	23·5	99	28·8	195	25·9
60–69	112	27·4	71	20·6	183	24·3
70 and over	107	26·2	51	14·8	158	21·0
Total	409	100·1	344	100·0	753	100·0

In all previously published studies a difference between the overall frequencies of atherosclerosis among cancer patients and controls has been reported. Such a difference is not found in the present study. Table IV shows the age-specific proportions with radiological evidence of aortic atherosclerosis for both males and females with and without a diagnosis of cancer. For each age and sex these percentages are essentially the same for patients with cancer and for patients without cancer. The age-adjusted percentages for males are 18·5 among the cancer patients and 18·7 among the non-cancer patients, and for females they are 24·8 for cancer patients and 23·5 for non-cancer patients. It is interesting that the percentage atherosclerotic for each age group among both the cancer and non-cancer patients is greater for females than for males with the exception of the non-cancer group aged 50–59. This is in partial agreement with the observation of Elkeles (1957) that atherosclerosis of the abdominal aorta is more common among older women than among men of the same age.

In Tables V and VI data for atherosclerosis of the thoracic aorta are presented according to sites and sex. Tables V and VI also indicate the difference between the age-adjusted percentages atherosclerotic for each of the site designations and

TABLE IV.—*Radiological Evidence of Calcification of the Thoracic Aorta According to Age, Sex, and Presence or Absence of Cancer*

Age	Cancer present			Cancer absent		
	Number observed	With calcification		Number observed	With calcification	
		Number	Per cent		Number	Per cent
Males						
40–49	46	2	4·3	49	—	—
50–59	110	11	10·0	65	8	12·3
60–69	131	27	20·6	68	15	22·1
70 and over	130	50	38·5	54	21	38·9
Total	417	90	21·6	236	44	18·6
Age adjusted*	—	—	18·5	—	—	18·7
Females						
40–49	94	7	7·4	123	6	4·9
50–59	96	14	14·6	99	11	11·1
60–69	112	27	24·1	71	21	29·6
70 and over	107	56	52·3	51	24	47·1
Total	409	104	25·4	344	62	18·0
Age adjusted*	—	—	24·8	—	—	23·5

* Adjusted to age distribution of all males and females.

for the non-cancer control group. No striking differences between the age-adjusted percentages atherosclerotic of patients with cancer of various sites and controls are noted. The largest difference (−10 per cent) is for white females with sites not specifically enumerated. On the other hand, for cancers of the buccal cavity and pharynx among males the difference is positive, that is, cancers exceed controls by 9 per cent. Of the 14 comparisons in both sexes, atherosclerosis is more frequent among cancer patients in 7 and less in 7.

TABLE V.—*Age-adjusted Percentages of Patients with Radiological Evidence of Calcification of Thoracic Aorta by Grouped Cancer Sites and Percent Difference Between Cancers and Controls, White Males*

Site	Int. list numbers	Number observed	With calcification			Differences in age-adjusted per cent, cancer patients minus controls
			Number	Per cent	Age-adjusted per cent	
Buccal cavity and pharynx	140–148	63	20	31·7	27·3	+8·6
Digestive organs and peritoneum	150–159	57	13	22·8	17·8	−0·9
Larynx, trachea, bronchus and lung	161–162	52	10	19·2	24·2	+5·5
Breast and genito-urinary	170–180	36	9	25·0	22·7	+4·0
Skin	190–191	104	16	15·4	13·0	−5·7
Lymphatic and Hematopoietic	200–205	27	4	14·8	17·3	−1·4
All other sites	—	78	18	23·1	18·5	−0·2

TABLE VI.—*Age-adjusted Percentages of Cancer Patients with Radiological Evidence of Calcification of Aorta According to Site and Percent Difference Between Cancer Groups and Controls, White Females*

Site	Int. List numbers	Number observed	With calcification			Differences in age-adjusted per cent, cancer patients minus controls
			Number	Per cent	Age-adjusted per cent	
Digestive organs and peritoneum	150–159	42	16	38·1	30·8	+7·3
Breast	170	117	35	29·9	30·3	+6·8
Cervix uteri	171	75	16	21·3	28·3	+4·8
Corpus uteri	172	27	8	29·6	27·3	+3·8
Other genito-urinary	173–181	24	8	33·3	19·9	−3·6
Skin	190–191	30	8	26·7	20·7	−2·8
All other sites	—	94	13	13·8	13·7	−9·8

According to Elkeles (1956), aortic atherosclerosis is particularly infrequent in patients with gastric carcinoma (7 per cent), carcinoma of the breast (14 per cent), and prostate (10 per cent). On the other hand, he indicates that the occurrence of aortic atherosclerosis is the same in patients with cancer of the respiratory tract (38 per cent) and controls (37 per cent). In the Roswell Park series there were 15 patients with cancer of the stomach, and of these 3 (20 per cent) showed evidence of aortic atherosclerosis. There were 117 patients with cancer of the breast, of whom 35 (30 per cent) showed evidence of atherosclerosis. There were 16 patients with cancer of the prostate and 5 (31 per cent) of these showed aortic atherosclerosis. Grouping cancer of the buccal cavity, pharynx, larynx, trachea, bronchus, and lung together yields 140 cases, of which 39 (28 per cent) had evidence of aortic atherosclerosis. These data do not support the contention that particular cancer sites are relatively free of aortic atherosclerosis.

In his studies of the dissociation between atherosclerosis and cancer, Elkeles (1956) used radiological evidence of calcification of the abdominal aorta as an index of atherosclerosis, since the abdominal aorta is usually the site of the earliest and most severe manifestations of aortic atherosclerosis. Since various portions of the arterial system show different atherosclerotic manifestations, frequently uncorrelated, our observations of the thoracic aorta are not really comparable with observations of the abdominal aorta. Of the 1462 patients in our sample, scout films of the abdomen were available on 509 (35 per cent). In addition, 115 abdominal radiograms were available for comparison with pathological diagnoses. In Table VII the radiological diagnosis of abdominal aortic calcification is compared with the pathological diagnosis of abdominal atherosclerosis. On the basis of routine scout films of the abdomen, the diagnosis of atherosclerosis of the abdominal aorta is shown to be somewhat less sensitive than for the thoracic aorta. Only 11 per cent of patients with the severest grades *i.e.* grades three and four, aortic atherosclerosis were discovered by radiological examination of the abdomen. Nevertheless, the technique was highly specific since none of the aortas graded zero or one by the pathologist was called atherosclerotic by the radiologist.

In Table VIII the frequencies of atherosclerosis of the abdominal aorta for cancer patients are compared with the control group. These data also failed to show any notable dissociation of aortic atherosclerosis between cancer patients

TABLE VII.—*Comparison of Radiological Determination of Calcification of the Abdominal Aorta and First Pathological Diagnosis of Aortic Atherosclerosis*

Radiological diagnosis	Graded pathological diagnosis									
	0		1		2		3		4	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Present	—	—	—	—	1	3·2	2	5·3	5	19·2
Absent	2	100·0	18	100·0	30	96·8	36	94·7	21	80·8
Total	2	100·0	18	100·0	31	100·0	38	100·0	26	100·0

and non-cancer patients. For both sexes combined, the difference between the age-adjusted percentages with atherosclerosis is 7 per cent, with cancer patients showing more evidence of atherosclerosis than controls. Unfortunately, the number of patients was not sufficient for comparisons by each cancer site.

TABLE VIII.—*Radiological Evidence of Calcification of Abdominal Aorta According to Sex and Presence or Absence of Cancer, Total Percentages and Age-adjusted* Percentages*

Sex	Cancer present				Cancer absent			
	Number observed	With calcification			Number observed	With calcification		
		Number	Per cent	Age-adjusted per cent		Number	Per cent	Age-adjusted per cent
Male	162	31	19·1	17·4	78	7	9·0	9·0
Female	174	25	14·4	15·5	95	9	9·5	10·2
Total	336	56	16·7	16·4	173	16	9·2	9·6

* Adjusted to age distribution of all males and females.

It is of interest to compare our findings and those of Elkeles (1956) with respect to calcification of the abdominal aorta among cancer patients. Since he published age-specific percentages, it has been possible to age-adjust his data and ours for comparison. When this was done, the frequency of aortic atherosclerosis among the cancer patients observed by Elkeles was the same as that observed at the Roswell Park Memorial Institute, namely, 16 per cent. We were also able to compare the percentages with atherosclerosis of the abdominal aorta in the control group in Elkeles' and the present series. Elkeles found atherosclerosis of the abdominal aorta among 35 per cent of his controls, while at the Roswell Park Memorial Institute 10 per cent showed atherosclerosis. These comparisons suggest that the differences between our findings and those of Elkeles may be due to differences in the types of patients used as controls. However, since Elkeles took special pains to diagnose calcification of the abdominal aorta, the observation of a similarity between the occurrence of this finding in his cancer series and ours may indicate an increased frequency of the condition in our series where no special effort was made to obtain radiograms for the diagnosis of abdominal aortic calcification.

DISCUSSION

The hypothesis that atherosclerosis is less frequent among patients with cancer than among controls, and that this dissociation varies according to site, interested us since it was consistent with certain endocrine hypotheses for the etiology of selected cancers and was consistent with certain concepts regarding the etiology of atherosclerosis. For example, Lilienfeld (1956) has shown that the excess of breast cancer in single women over married women may be attributed to the later entry into the menopausal state of single women and that this difference in age may be due to the increased frequency of artificial menopause in married women. Winkelstein, Stenchever and Lilienfeld (1958) have shown that women with a history of myocardial infarction have a more frequent history of abortion and artificial menopause than a control group. These two observations suggest the hypothesis that breast cancer and myocardial infarction are dissociated and would lead one to expect that breast cancer and atherosclerosis are likewise dissociated. This is consistent with other data suggesting that estrogens have a protective effect with respect to coronary atherosclerosis and may serve as a predisposing influence on the development of breast cancer (Stamler, Katz, Pick and Rodbard, 1955 ; Wuest, Dry and Edwards, 1953 ; Rivin and Dimitroff, 1954). On the other hand, it has been shown that coronary atherosclerosis is not necessarily correlated with aortic atherosclerosis (Epstein, Boas and Simpson, 1957 ; Pick, Stamler, Rodboard and Katz, 1952) so that failure to show a dissociation between aortic atherosclerosis and cancer of the breast does not shed light on the hypothesis that breast cancer and myocardial infarction are dissociated.

The observations reported here which indicate that there is no dissociation between cancer in general, or cancer of particular sites, and atherosclerosis of the thoracic aorta are in sharp contrast to previously published data. Nevertheless, the frequency of atherosclerosis of the abdominal aorta among the cancer patients surveyed in this study is similar to that observed by at least one previous investigator (Elkeles, 1956). It is our feeling that the differences observed in previous studies stem from two causes. In studies of autopsied persons, cancer patients have been subjected to a variable period of starvation and malnutrition prior to death which may have produced a remission in the severity and frequency of atherosclerotic lesions. Furthermore, control material drawn from autopsy studies would tend to be weighted by cases in which atherosclerosis might be a frequent accompaniment. The second factor, i.e., selection, may also be responsible for the differences observed in the radiological studies. One would expect hospital populations to contain larger proportions of hypertensive and diabetic patients than the general population. Epstein, Boas and Simpson (1957) have shown that diabetes mellitus is associated with an increased frequency of calcification of the aorta, determined radiologically, when compared with a control group without diabetes.

It would seem that the utilization of patients suspected of cancer but subsequently shown to be without this disease as controls would eliminate some of the selective factors likely to be present in the study of a general hospital population. It is unlikely that diseases such as diabetes and hypertension which are known to predispose patients to the development of aortic atherosclerosis would be selectively preferred among patients with suspected cancer, whereas they

would be expected to be more frequently represented in general hospital admissions than in the general population.

It must be admitted that the difference in results between this study and that of Elkeles may reflect other factors whose frequencies differ in the United States and England. However, we think that this is an unlikely explanation.

Unfortunately, the present study does not adequately answer the question as to whether there exists a dissociation between the occurrence of coronary artery disease and cancer. This more fundamental problem probably requires a prospective study. A satisfactory answer could only be obtained by observing the occurrence of manifest coronary disease in a group of patients with various types of cancer and in a control group followed prospectively. Comparison then of the frequencies of coronary disease in these groups would provide the answer with respect to the dissociation hypothesis.

SUMMARY AND CONCLUSIONS

1. The frequency of atherosclerosis of the thoracic and abdominal aorta has been determined radiologically in a group of cancer patients and non-cancer patients.

2. Posterior-anterior thoracic radiograms are highly specific in indicating the presence of calcification of the aorta while scout films of the abdomen are somewhat less sensitive but also highly specific.

3. The frequency of calcification of the thoracic aorta and of the abdominal aorta has been shown to be essentially the same in cancer and non-cancer patients. The percentages of cancer patients with radiological evidence of atherosclerosis of the aorta is essentially the same for various types and sites of cancer.

4. The previously reported observation that there is a dissociation between atherosclerosis of the aorta in cancer patients and non-cancer controls is not confirmed in the present study.

The authors wish to express their appreciation to Dr. J. E. Dowd and Dr. S. Rodbard for their comments and suggestions and to Dr. Shale Brownstein for his assistance in collection of data.

This study was aided in part by a grant from the American Cancer Society.

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A CASE OF PRIMARY OSTEOGENIC SARCOMA OF EXTRASKELETAL SOFT TISSUES

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Received for publication September 29, 1959

METAPLASTIC bone formation in benign mesenchymal tumours is occasionally seen, as for example in fibromas and synoviomas (Evans, 1956; Willis, 1948), and in lipomas (Plaut, Salm and Truscott, 1959). Ossifying malignant neoplasms outside the skeleton have been recorded in many organs, but malignant osteogenic tumours arising primarily in the soft tissues are exceedingly rare. Fine and Stout (1956), who reviewed this subject, found only one such case amongst their own material of over 156,000 specimens. They were able to trace 34 primary osteogenic sarcomas of soft tissue in the literature, to which they added their own and a further 11 referred cases, making a total of 46 cases. Yet in only 9 of these does the record include a post-mortem examination. It is the purpose of this paper to present a complete record of such a case who was followed up closely since his first attendance.

Case History

The patient, a farmer aged 51 years, presented himself in February 1958 with a large, raised, fungating mass above his left iliac crest. He had been aware of it for the past 18 months and decided to seek medical advice only after seeing a television series eulogizing medical skill. No evidence of metastatic spread was found in inguinal lymph glands and lungs. Radiologically the growth appeared to contain bone, but was not connected with the skeleton. The tumour was easily excised; it was embedded entirely in the soft tisues of the left loin and nowhere attached to or near to the iliac bone. His wound healed well, but in July 1958 he had a haemoptysis and small secondaries were visualised for the first time on radiological examination of his lungs. He soon started to lose weight, continued to have haemoptyses and his pulmonary secondaries steadily enlarged. Left-sided inguinal glandular metastases developed from October 1958 onwards, ultimately attaining a large size with erosion of the overlying skin. In January 1959 the serum alkaline phosphatase was found to be raised to 28 units. His condition continued to deteriorate and he died of cachexia in August 1959, 18 months after excision of the primary growth and after a total illness of about 3 years.

Operation specimen

The excised mass measured 15 × 9½ × 8 cm. and was covered at one aspec- by a spindle-shaped piece of skin. This was bearing a broadly sessile, dark brown, patchily haemorrhagic tumour, measuring 6 cm. in length and raised

4 cm. above the skin surface. On slicing the growth was seen to extend up to 6 cm. into the underlying fat tissue (Fig. 1). The excision had just cleared its lower margin. Parts were necrotic. The better preserved areas were greyish in colour and firm-elastic in consistency. Several small scattered bony areas were noted as well as one large, excentrically placed, solid bony mass, measuring $5 \times 3\frac{1}{2}$ cm., which necessitated sawing.

Necropsy

This was performed 10 hours after death. The operation scar was free from recurrence. Large, necrotic glandular masses were present in the left groin, covered by ulcerated skin and situated entirely within the inguinal soft tissues Both lungs contained many secondary deposits (Fig. 2), measuring from 2 to 10 cm. across, which varied in consistency from soft to that of cartilage, and some were almost solid bone. An occasional pulmonary metastasis had grown into the bronchial lumen in polypoidal fashion causing partial obstruction. The hilar lymph glands were not involved. The pancreas contained 3 small ossified metastases measuring 1 cm. in diameter, and a single larger, soft secondary, measuring $3\frac{1}{2}$ cm. across. Five pedunculated or broadly sessile metastases were found in the lower half of the duodenum and upper jejunum (Fig. 3), measuring from 1 to 5 cm. across ; two were hard and one of these had to be bisected with the help of a saw. The brain contained a single, small, depressed metastasis in the cortex of the right fronto-parietal region, and a further small pedunculated deposit was found on the right parietal pleura. The skeleton was not involved ; in particular a detailed examination of vertebral column, sacrum, wing of left ileum and left pubic ramus failed to show any evidence of tumour growth.

An alkaline phosphatase estimation, using heart blood, gave a reading of 140 units.

Histology

Five blocks were cut from the operation specimen and 28 from the necropsy material. The microscopical features of both primary growth and secondary deposits were very similar. In the soft areas the tumour had the appearance of a rapidly growing pleomorphic sarcoma with many tumour giant cells and abnormal mitoses, growing either in diffuse sheets or displaying a fasciculated arrangement (Fig. 4). In many sites the growing margins were sharply defined (Fig. 5), in others the neoplasm infiltrated diffusely into the surrounding subcutaneous fat or adjacent alveoli. The presence of intrabronchial growth was confirmed ; the bronchial walls had been replaced, but the bronchial cartilage and epithelium tended to be spared (Fig. 6). In the original growth as well as in many secondaries of lung and in the pancreas the tumour showed much osteoid tissue and bone, particularly dense bone having been formed by one of the pancreatic metastases (Fig. 7 and 8). Frequently the bony trabeculae were invested with rows of malignant osteoblasts (Fig. 9). In addition small areas of malignant cartilage were also in evidence and some of these were undergoing ossification at their margins. Transformation of sarcomatous tissue into malignant cartilage was noted in many areas. Where this was present adjacent to normal bronchial cartilage the benign and malignant cartilaginous tissues were easily distinguishable from each other.

DISCUSSION

The present case appears to conform with many features brought out by Fine and Stout in their 1956 review. The average age of sufferers from extraskeletal osteogenic sarcoma of soft tissues is higher than that of osteosarcoma of bone and most patients are over 30 years of age. On the whole males appear to be affected more often than females and the extremities are involved more often than the trunk. Most tumours proved to be very malignant, the average 5-year cure being a little less than 9 per cent. Fine and Stout made the arbitrary distinction between osteolytic and osteogenic types, but emphasise that the quantity of bone present and the numbers of mitoses do not indicate the degree of malignancy nor the likelihood of metastases. In the present case anaplastic and osteogenic areas alternated with each other both in the primary growth and in the metastases. The lungs are the most common site of secondary spread, but in several instances widespread dissemination was recorded. Formation of malignant cartilage was noted in several instances.

Osseous metaplasia is known to occur in reparative tissue, myositis ossificans, skin and other sites, and has been found in benign mesenchymal tumours, in the stroma of epithelial neoplasms and also in primary malignant mesenchymal growths. In all these tumours the tissues had no connection with bone and the sites precluded the presence of avulsed periosteum or osteoblasts. Bone formation must be regarded as due to divergent differentiation of the mesenchymal tumour cells themselves. This is in no way surprising. Whilst, as Willis (1948) has pointed out, the cell type of several kinds of mesenchymal tissue tends to be perpetuated in their tumours, when cells of mesenchymal origin multiply they can reacquire embryonic properties and redifferentiate in aberrant directions. Thus, in extraskeletal osteogenic sarcoma the proliferating tumour cells do not go on to produce, for example, collagen but differentiate into cartilage, osteoid and bone. This pluripotential property of the proliferating mesenchymal cell is most likely also the explanation of the so-called mesenchymoma, a term used to designate benign and malignant neoplasms made up of two or more types of mesenchymal tissue (Stout, 1948 ; Symmers and Nangle, 1957).

EXPLANATION OF PLATES

FIG. 1.—Cross-section through soft part of the primary growth showing haemorrhagic appearance and large "submerged" part. Small part projecting above skin surface. About × ½.

FIG. 2.—Cut surface of left lung showing multiple secondary deposits. About × ⅓.

FIG. 3.—Two polypoidal jejunal metastases. Slightly reduced.

FIG. 4.—Primary growth. Anaplastic sarcomatous area with tumour giant cells. Haematoxylin and eosin. × 90.

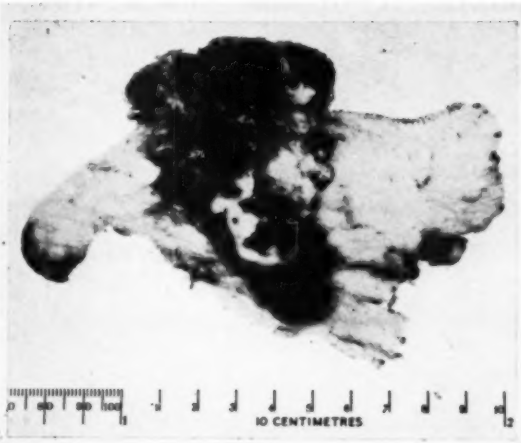
FIG. 5.—Primary growth. Fairly sharp demarcation towards subcutaneous fat (bottom). Malignant cartilage at top left, osteoid formation at bottom right. Haematoxylin and eosin. × 60.

FIG. 6.—Pulmonary metastasis. Bronchial cartilage enveloped by anaplastic sarcoma. The bronchial epithelial lining is still largely intact. Above : malignant cartilage and calcifying osteoid. Smaller islet of malignant cartilage below. Haematoxylin and eosin. × 60.

FIG. 7.—Pancreatic metastasis showing dense bone formation. Malignant osteoblasts in lacunae. Haematoxylin and eosin. × 60.

FIG. 8.—Pancreatic metastasis. Trabecular osteoid pattern. Haematoxylin and eosin. × 60.

FIG. 9.—Primary growth. Ossifying osteoid trabeculae with malignant cartilage at top. Haematoxylin and eosin. × 60.



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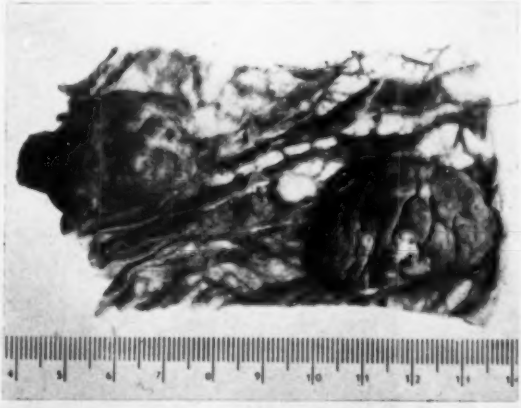

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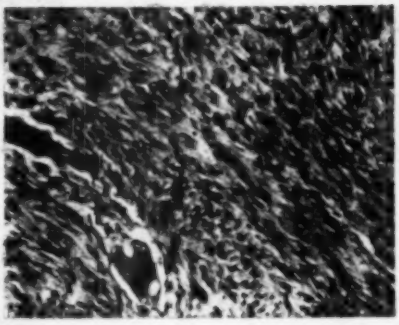

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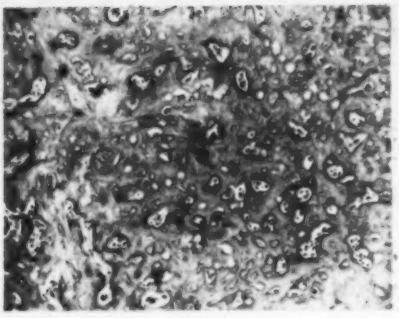

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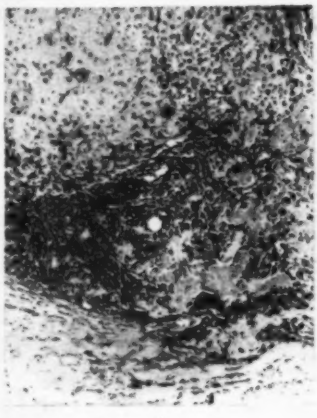

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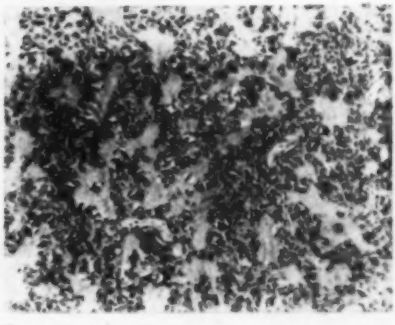

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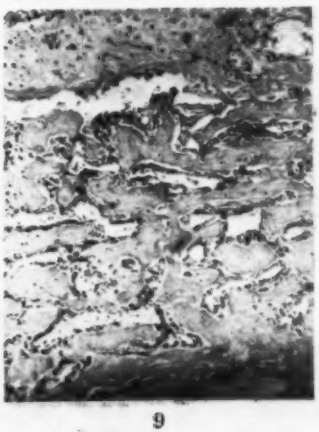

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Salm.

But although the histogenesis of osteogenic sarcoma of soft tissues can be readily explained along these lines, they are uncommon tumours and as such worthy of note.

SUMMARY

A case of osteogenic sarcoma of the extraskeletal soft tissues of the loin is recorded, with widespread bone-forming metastases studied at necropsy.

The histogenesis of this type of growth is discussed and it is concluded that these tumours arise due to aberrant differentiation of the multiplying malignant mesenchymal cell.

My sincere thanks are due to Professor R. A. Willis for confirming the diagnosis and reading the manuscript. I am indebted to Mr. T. M. Reid for the surgical details and to Dr. C. B. O'Carroll, the patient's practioner, for his help and co-operation. I am grateful to Miss Phyllis E. Coleman for the photographic work.

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3 : 4-BENZPYRENE IN INDUSTRIAL AIR POLLUTION : SOME REFLEXIONS

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Received for publication July 30, 1959

AMONG the known carcinogens producing lung carcinoma in man : arsenic, asbestos, beryllium, chromium, nickel and ionizing agents can account for only a small fraction of all cases occurring, because these agents are very limited in their distributions.

As 3 : 4-benzpyrene, a well known experimental carcinogen for epidermis and connective tissue, has been found in tobacco smoke (Cooper, Lindsay and Waller, 1954 ; Cooper and Lindsay, 1955 ; Seelkopf, 1955) and in the air of populated areas, especially town air (Waller, 1952 ; Kotin, Falk, Mader and Thomas, 1954 ; Stocks and Campbell, 1955 ; Campbell and Clemmesen, 1956 ; Campbell and Kreyberg, 1956), the view has been advanced by numerous students, that 3 : 4-benzpyrene is the important factor in the increase in lung carcinomas in recent years, especially among males in towns.

Stocks and Campbell in their paper even attempted to calculate the respective role of 3 : 4-benzpyrene from the two sources. Such calculations are, however, of restricted interest because the cases of lung tumours used for the calculations were mainly based upon death certificates and the histological typing ignored. The latter point is of special importance when the absolute number of cases is low, as is the case in rural districts in Norway (Kreyberg, 1959). It may be of considerably lesser importance in England and Wales, where the lung cancer incidence is high, even in rural districts.

In the rural areas not only the amount of 3 : 4-benzpyrene in the general air is lower than in more densely populated areas and towns, but the number of special factories and industrial plants producing carcinogens are also fewer. A certain consideration should therefore be paid, when lung cancer frequency in urban and rural areas are compared, to the occupation of the victims.

It may be that some of the lung cancer patients in the rural districts have been exposed to important concentrations of carcinogens during their working hours, in spite of living in an air little polluted generally. With the increasing dispersion of industry in countries with ample access to water-power (and in the future to atomic power) an increasing population will work in factories with polluted air, in areas administratively designated as " rural ".

Under these circumstances a study of the occurrence of 3 : 4-benzpyrene in industrial plants should be of some interest.

Gasworks naturally present themselves for study, because gas fumes contain benzpyrene, and statistical evidence points in the direction that lung cancer occurs more frequently among gas workers, than in the general population (Kuroda and Kawahata, 1936 ; Kennaway and Kennaway, 1947 ; Doll, 1952).

Another source of air, polluted with 3 : 4-benzpyrene, came to my attention some years ago, when a paper by Livingston (1953) described warts appearing on the hands, arms, ankles and face of workers connected with the Søderberg furnace in an aluminium factory in England.

At the same time I had the opportunity to examine a tar developed during aluminium production in a Norwegian factory. The tar showed a very high carcinogenic potency when painted on the skin of mice, and by chemical analysis the tar was shown to contain 1 per cent 3 : 4-benzpyrene.

During the aluminium production fumes from such tars will in varying amount pollute the air of some parts of the factory.

On this background I sought contact with the gasworks of Bergen (director B. Paulson) and Oslo (director W. Eckhoff), as well as with the Norwegian State-owned and controlled aluminium factories (director Aa. W. Owe).

The two directors of the gasworks readily gave their full support. The director of the aluminium factories, however, flatly refused co-operation on similar terms. My application for permission to study the air in the privately owned A/S Elektrokemisk, which holds the rights of the Søderberg electrode was, however, immediately generously granted by the director-general G. Hagerup Larssen, and the director of Fiskaa Verk J. Gørrissen, and all facilities extended.

Gasmeters equipped with filter papers were installed in the plants at representative sites where the workers would be regularly employed, as described in a previous paper (Campbell and Kreyberg, 1956).

The filter papers were most kindly examined by Mr. B. T. Commins at the "Group for Research on Atmospheric Pollution", St. Bartholomew's Hospital, London.

The results of the investigations of the three industrial plants are summarized in Table I. For comparison figures from the air of some towns in England and Wales are given, quoted from Commins (1958).

TABLE I.—*The Content of Hydrocarbons in the Air in μg. per 100 m³*

	Source of sample					
	Industrial Plants			General Town Air		
Hydrocarbon	Oslo Gasworks	Bergen Gasworks	Elektrokemisk	Liverpool	Wrexham	Llangefni
	(μg. per 100 m³)					
Fluoranthene	1300	240	100	6·7	2·0	0·4
1 : 2-benzanthracene	1400	280	80	..	..	..
Phenanthrene	Not detected	8	27	..	..	..
1 : 2-benzpyrene	150	130	10	..	..	..
Pyrene	870	240	69	5·0	1·8	0·3
Coronene	20	30	Trace	..	..	..
1 : 2-benzperylene	210	200	5	16·6	5·1	0·5
3 : 4-BENZPYRENE	730	200	18	6·8	2·0	0·4
Anthracene	100	Trace	Present	..	0·4	0·1
Anthrathene	80	60	Trace	..	..	..
Fluorene	20	Trace	,,	..	..	..
Naphthalene	Not detected	Not detected	..	..	..	..

For further comparison, the seasonal changes of 3 : 4-benzpyrene in the air in towns of England and Wales and in Norway are presented in Fig. 1, quoted from the paper of Campbell and Kreyberg (1956).

The first comment to these findings is to stress that they do not at all intend to give a complete picture of the air pollution in such plants. The report actually represents a pilot study and should be followed up with more extensive and more complete investigations.

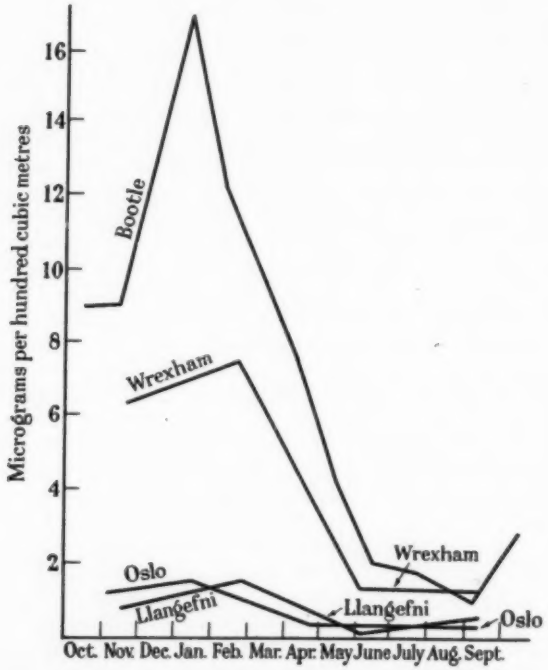

FIG. 1.—Yearly concentration of 3 : 4-benzpyrene in the air at Oslo in 1955 compared with Bootle, Wrexham and Llangefni in England and Wales (Campbell and Kreyberg, 1956).

With the coarse technique used, the figures indicate that the air in the retort house of Oslo Gaswork contains 3 : 4-benzpyrene in amounts corresponding to some 5000 cigarettes daily for a worker with a 40 hours week. Even if the figures for Bergen are lower, they are nevertheless of the same order of magnitude and the " cigarette-equivalent " is enormous.

From the study of cases of human lung cancer two observations are of interest in this connection : 1. There seems to be a straight line correlation between the number of cigarettes smoked and the risk of developing epidermoid and small cell anaplastic carcinomas. In an English material the increase in risk is some 25 times after regular daily smoking of 30 cigarettes (Doll, Hill and Kreyberg, 1957). 2. The risk among gas workers to develop lung cancer is in the literature

(Doll, 1952) ranged between twice and ten times the risk of the general population, smokers included.

If these two facts are correlated : the enormous amounts of 3 : 4-benzpyrene in the air and the very moderate excess of lung cancer in the gasworks, a serious fallacy is evidently involved. One explanation of the discrepancy between amount of 3 : 4-benzpyrene in the air and risk of lung cancer may be the following :

The benzpyrene is suspended in the air as finer or coarser particles adsorbed to other particles, particularly flakes of soot. Biologically active is only that fraction which enters the body, in the present case the bronchial linings.

Besides, the physico-chemical measurement of the substance under suspicion, also knowledge of the particle size is necessary, as only particles under and above a certain size are deposited in any numbers on the bronchial linings.

This represents a serious warning against any conclusions as to causative relationships between any substance and lung cancer based upon the mere finding of the substance deposited on paper filtering the air.

Another explanation of the lack of correspondence between the amounts of 3 : 4-benzpyrene in the air and the number of lung cancer cases in the gasworks may be that there is no causative relationship at all.

The overwhelming evidence to-day shows that the rise in frequency in epidermoid and small cell anaplastic lung carcinomas is mainly caused by cigarette smoking. The active agent in the cigarette smoke is not known.

The main support for the assumption that 3 : 4-benzpyrene is an important factor in the increased development of lung cancer is its immediate plausibility. The substance is present in polluted town air and in tobacco smoke, and the substance is carcinogenic in animals.

But 3 : 4-benzpyrene is present in cigar and pipe smoke in even greater concentrations than in cigarette smoke (Cardon, Alvord, Rand and Hitchcock, 1956 ; Gilbert and Lindsay, 1956), but not connected with anything like the same risk for lung cancer development. The role of 3 : 4-benzpyrene in the development of lung cancer is very far from known.

The main purpose of publishing these sparse data is to enter a plea for a more complete technique for future studies of possible carcinogens in the air inhaled by the human population. The particle size should be recorded in addition to the statements of the amounts present.

I wish to express my sincere thanks to the Directors B. Paulson of Bergen Gaswork, W. Eckhoff of Oslo Gaswork, and G. Hagerup Larssen and J. Gørrisen of A/S Elektrokemisk for their kind help, readily given.

This investigation was aided by a generous grant from Aktieselskapet Borregaards Forskningsfond for which I express my warmest thanks.

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INCOMPLETE CARCINOGENS IN CIGARETTE SMOKE CONDENSATE: TUMOUR-PROMOTION BY A PHENOLIC FRACTION

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Received for publication November 5, 1959

The carcinogenicity of tobacco smoke condensate for mouse skin, which was demonstrated by Wynder and his colleagues in 1953 and subsequently (Wynder, Graham and Cronnger, 1953; and Wynder, 1959 for review), has now been confirmed by several other groups of workers (Engelbreth-Holm and Ahlmann, 1957; Orris *et al.*, 1958; and Bock and Moore, 1959). Passey (1957) also observed benign and malignant skin tumours in mice painted with tobacco smoke condensate. He pointed out, however, that the mice, which were of one of the strains used by Wynder and his colleagues, developed spontaneous ulcers of the skin. These appeared in untreated and treated mice alike, and the author considered that their occurrence rendered interpretation of the results difficult. In all these experiments the smoke condensate was applied at short intervals for long periods.

The possibility that the two stage mechanism of carcinogenesis might be involved was early considered. Wynder *et al.* (1953) included an experiment in which tobacco smoke condensate was applied together with the tumour-promoting agent, croton oil, to mouse skin. The yield of tumours was no greater than with smoke condensate alone. Gwynn and Salaman (1956) examined whole smoke condensate for the presence of incomplete carcinogens. They tested the condensate for both tumour-initiating and tumour-promoting activity, and obtained some evidence of promotion but none of initiation. The results of Hamer and Woodhouse (1956) also suggested that smoke condensate has tumour-promoting action, but they obtained no evidence for tumour initiation, or for complete carcinogenicity. Gellhorn (1958), in several large scale experiments, demonstrated very convincingly the tumour-promoting effect of whole smoke condensate.

Wynder at a meeting at the Chester Beatty Research Institute on July 6, 1959 spoke of evidence which his group had obtained for tumour-promotion by the nicotine-free basic fraction of smoke condensate.

The experiments to be described are a further stage in the examination of tobacco smoke condensate for incomplete carcinogens (tumour initiators and promoters). The strong promoting action of one fraction of the condensate is reported.

MATERIALS AND METHODS

Mice.—Male and female mice of the " 101 " inbred strain were used. They have been maintained by brother-sister mating in this department since a breeding pair was obtained from Dr. T. C. Carter (Radiobiological Research Unit, A.E.R.E., Harwell, Didcot, Berks) in November, 1955.

This strain was selected for its high and uniform susceptibility to skin tumour-induction by initiating and promoting agents (Salaman, 1956). Not until half way through the experiments was it realized that the mice are not very suitable for long term experiments because of a high incidence of a renal disease, papillo-nephritis, first observed in Strain A mice by Gorer (1940), and subsequently studied in greater detail by Dunn (1944). As in strain A mice, the disease shortens the life span of affected animals so that few live as long as 18 months.

Mice were fed on cubes according to the Rowett Institute formula (Thomson, 1936) and water *ad libitum*.

Before the experiment mice were vaccinated on the tail with sheep lymph as a precaution against ectromelia ; all responded with a typical primary response.

The dorsal hair was removed by electric clippers at the beginning of the experiment and thereafter as necessary.

Preparation of cigarette smoke condensate and its fractions

These were kindly supplied by the Research Laboratories of the Tobacco Manufacturers' Standing Committee.

Details of the preparations are given by Mr. J. G. Burgan in an appendix to this paper.

Freshly prepared samples of whole smoke condensate and its neutral fraction were mailed to us each week, and were used within two weeks of receipt. Storage between arrival and use was in small sealed ampoules kept protected from light at 4° C.

Large samples of the smoke phenol fraction were mailed to us at 6-weekly intervals, and stored similarly before use.

Application of materials to the skin

9,10-Dimethyl-1,2-benzanthracene (DMBA), obtained from L. Light & Co., was applied as a 0·15 per cent solution in acetone with a graduated pipette.

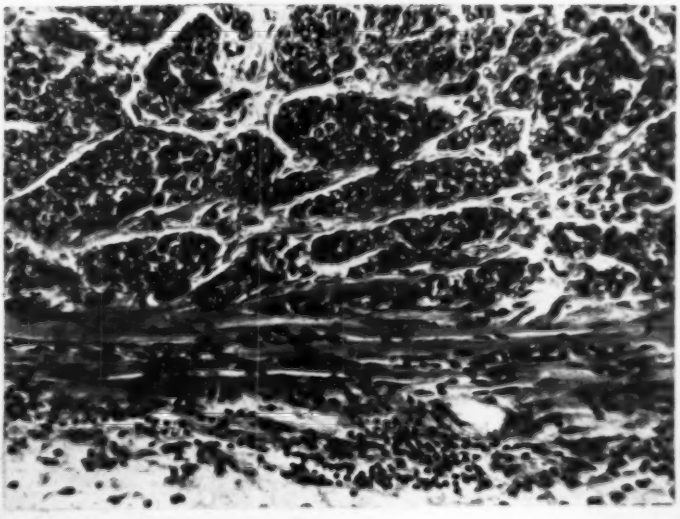
All the smoke condensate fractions were too viscous, even after dilution with acetone, to be applied quantitatively with a graduated pipette, or by a wide-mouthed dropper : a brushing-on technique was necessary. The whole of the back from the neck to the root of the tail was painted, using a squirrel-hair brush.

EXPLANATION OF PLATE

FIG. 1.—Papillomata on the back of a mouse treated once with DMBA (300 μg.) and then thrice weekly with the phenolic fraction of cigarette smoke condensate (6–12 mg. per application) for 27 weeks.

FIG. 2.—Photomicrograph of a malignant tumour which arose on the dorsal skin of a mouse similarly treated with phenolic fraction for 41 weeks after a single application of DMBA. Invasion of the panniculus carnosus muscle can be seen. (Staining : haematoxylin and eosin. Magnification : × 230.)

1



2

The whole smoke condensate and neutral fractions as received were diluted 3 or 4 times with acetone. After each painting session the average amount of condensate or fraction applied to each mouse was calculated retrospectively as follows :—

$$\text{Average weight of material applied to each mouse} = \frac{\text{Wt. of fraction + acetone used}}{\text{No. of mice painted}} \times \text{per cent fraction in fraction-acetone mixture.}$$

With experience it was possible to keep this average to within ± 20 per cent of the intended dose.

The smoke phenol fraction was diluted with acetone 1 in 4, 1 in 6, or 1 in 8 according to dose level. It was found that painting the clipped skin with the strongest of these concentrations left on the average 12 mg. of smoke phenols on the mouse ; 1 in 6 left 9 mg., and 1 in 8 left 6 mg.

The acetone used was of the Analar grade supplied by British Drug Houses.

Histological examination.—Biopsy specimens, and tissues taken for section post mortem, were fixed in Zenker's fluid, embedded in paraffin wax, and stained with haematoxylin and eosin.

EXPERIMENTAL

Experimental I : whole smoke condensate

Thirty male and 32 female mice were clipped, and painted thrice weekly with whole smoke condensate. At first approximately 25 mg. was applied to each mouse at each application. This dose was steadily increased during the first 3 weeks up to an average of 40 mg. per mouse, and thereafter maintained at approximately this level until the 30th week. It was then reduced, because of toxic effects, to 30 mg. per application.

Table I indicates the progress of the experiment. Fifty-two mice died during the first 40 weeks : gross pathological changes indicative of papillonephritis (see p. 624) were present in 13 mice (this incidence does not differ significantly from that in untreated mice), pneumonic consolidation in 6, intussusception in 3, enteritis in 3. Nineteen mice died during the 24th week from the toxic effects of an unusually large dose of smoke condensate applied in error. Several other mice which showed no obvious lesions, and some of those with only slight kidney lesions, probably died from the cumulative toxic effects of the condensate. Advanced post mortem changes prevented satisfactory examination in 11 mice.

One papilloma appeared during the 37th week, when there were only 13 survivors. The same mouse developed a second papilloma during the 44th week, but died two weeks later. No other tumours have appeared. There are still 3 mice alive after 60 weeks of treatment but none has any skin tumours.

Experiment II : Neutral fraction

Thirty male and 33 female mice were clipped and painted thrice weekly with the neutral fraction of tobacco smoke. At first approximately 25 mg. was applied to each mouse at each application. This dose was steadily increased during the first 3 weeks up to an average of 40 mg. per mouse. This level was thereafter maintained.

TABLE I.—*Tests of Whole Smoke Condensate and its Neutral Fraction for Carcinogenicity on Mouse Skin*

Time (in weeks)	Total weight of test material applied* (g.)	Survivors	Number of mice with papillomas	Total papillomas	Total carcinomas
		Experiment I : Whole Smoke Condensate			
0	—	62	—	—	—
5	0·50	62	—	—	—
10	1·10	62	—	—	—
15	1·66	59	—	—	—
20	2·22	50	—	—	—
25	2·72	30	—	—	—
30	3·17	22	—	—	—
35	3·52	13	1	1	—
40	3·96	11	2	2	0
		Experiment II : Neutral Fraction			
0	—	63	—	—	—
5	0·50	60	—	—	—
10	1·10	59	—	—	—
15	1·66	58	—	—	—
20	2·22	46	—	—	—
25	2·78	44	—	—	—
30	3·38	37	—	—	—
35	3·90	25	3	4	—
40	4·46	17	1	1	0

* In both experiments the test material was applied thrice weekly throughout. At first 25 mg. amounts, diluted 3 or 4 times in acetone, were applied on each occasion. This dose was steadily increased to 40 mg. per application during the first 3 weeks of treatment. In the case of the neutral fraction the dose level was maintained at 40 mg. throughout the experiment, but in the case of whole smoke condensate it was reduced to 30 mg. per application after 30 weeks because of systemic toxicity.

Table I indicates the progress of Experiment II. Thirty-eight mice died during the first 40 weeks. Compared with the mice treated with whole smoke condensate, pneumonic consolidation (12 cases) was more frequent in these mice. Tyzzer's disease caused 3 deaths, intussusception 3, and enteritis a further 4. Papillonephritis was seen in 18 mice. One mouse died as a result of haemorrhage from a tumour of the tongue. On histological examination this tumour was of squamous cell type, it showed doubtful invasion of muscle and no metastases. It was regarded as probably, but not certainly, malignant. Advanced post mortem changes prevented satisfactory examination in 10 mice.

Two papillomas appeared during the 32nd week of the experiment, one in a mouse which died 10 days later (post mortem decay prevented satisfactory examination), and the other in a mouse which died 4 weeks later and showed marked renal changes due to papillonephritis. Whilst under observation, both tumours were notably slow-growing. A third mouse developed a papilloma during the 40th week of the experiment. Treatment was stopped after 47 weeks. Three weeks later two of the 10 survivors (both females) developed 2 papillomas each. These grew more rapidly than those which arose earlier, and soon one of the tumours on each mouse took on the naked-eye appearances of malignancy. One was removed at biopsy during the 60th week and was found to be a squamous-cell carcinoma invading muscle.

Experiment III : Smoke phenols fraction

One hundred and ten mice, divided into 3 groups, were used in this test. Each group contained equal numbers of males and females. Groups I and II consisted of 30 mice each, and Group III of 50 mice. Groups I and III were given a single application of 0·2 ml. 0·15 per cent DMBA in acetone to the clipped dorsal skin. Group II was not treated at this time. Three weeks later groups I and II began a course of thrice weekly applications of smoke phenols fraction in acetone. For the first 9 applications a 25 per cent solution/suspension of the fraction in acetone (i.e. average of 12 mg. smoke phenols per mouse per application) were given. The skins of many animals became thick and crusted, and 3 mice died on the 19th day as a result of systemic toxicity. The concentration was therefore reduced to 12·5 per cent and continued at this level for a further 15 applications. It was then increased to 18·7 per cent for 24 applications, and then back to 25 per cent from the 49th application (17th week) onwards. Group III received no treatment apart from the single application of DMBA.

Of the 10 mice of Group I which died during the first 40 weeks of the experiment, i.e. 37 weeks of treatment with smoke phenol fraction, one had lymphatic leukaemia with enlargement of the thymus and lymph glands, one had a large mesenteric tumour (unfortunately post mortem changes were too advanced for satisfactory histological examination) 2 showed pneumonic consolidation, and 1 had 2 small lung adenomas. Four had abscesses in the liver and 3 showed papillonephritis.

Of Group II, 14 mice died. As mentioned above, 3 died early (19th day) from systemic toxic effects. A large abdominal tumour was seen in a female mouse which died during the 37th week. This tumour was adherent to the spleen, pancreas and mesentery. Histological examination showed it to be a haemangioma. This mouse was one of 3 which had liver abscesses. Three mice showed pneumonic consolidation, and 7 had papillonephritis.

In Group III 13 mice died and 7 of these were examined post mortem. One had lymphatic leukaemia with a grossly enlarged thymus and one had 2 small lung adenomas. One mouse had intussusception, one bronchopneumonia, and one a large abscess of the neck. Five of the 7 showed kidney lesions indicative of papillonephritis. Advanced post mortem changes prevented satisfactory examination of the remaining 6 mice.

Incidence of skin tumours

Papillomas began to arise in mice of Group I during the 15th week. By the 37th week of treatment with the phenolic fraction 15 out of the 20 survivors of this group bore a total of 65 papillomas (see Table II and Fig. 1). All these tumours arose on the treated area of skin, and none were seen outside it. At the 37th week one tumour on a male showed macroscopic features suggestive of malignancy. During the next 4 weeks this tumour grew quickly, ulcerated, and became adherent to deeper structures. It was removed by biopsy during the 41st week of the experiment, and histological examination showed it to be a poorly differentiated carcinoma which had penetrated the panniculus carnosus muscle (Fig. 2).

Treatment with smoke phenol fraction was stopped after 41 weeks (44th week of experiment). The mice have been kept under observation ; a further probably malignant tumour has arisen on another mouse (a female) but no specimen has yet been taken for histological examination.

TABLE II.—*Tests of the Phenolic Fraction of Cigarette Smoke Condensate for Carcinogenicity, and for Tumour-promotion after a Single Application of DMBA, on Mouse Skin*

Group	Number of mice	Treatment	Tumour incidence at 40 weeks*			
			Survivors	Mice with papillomas	Total papillomas	Total carcinomas
I	30	300 μg. DMBA followed, after 3 weeks interval, by thrice weekly applications of smoke phenols fraction†	20	15	65	1
II	30	Thrice weekly applications of smoke phenols fraction only	16	0	0	0
III	50	300 μg. DMBA only	37	4	6‡	0

* i.e. After 37 weeks of smoke phenols treatment.

† The smoke phenols fraction was diluted with acetone 1 : 4 to 1 : 8, depending on dose level (see text, p. 625). The first 9 applications of smoke phenols fraction were of 12 mg. each; the next 15 were of 6 mg.; the next 24 of 9 mg.; and thereafter all applications were of 12 mg. each.

‡ All outside the treated area (see text, p. 000).

No tumours have appeared on mice of Group II.

Four mice in Group III developed a total of 6 papillomas during the 40 weeks after treatment with DMBA. All these tumours were situated on the head: no tumours arose on the treated dorsal skin. It is odd that no such ectopic tumours arose in Group I mice which received similar treatment with DMBA as well as treatment with smoke phenols fraction. A similar result was obtained in this laboratory, in "S" strain mice: treatment with DMBA alone gave rise to many ectopic tumours, whereas DMBA followed by repeated applications of croton oil gave rise to very few (Roe, 1956).

Whatever the explanation of this curious phenomenon, the tumour-incidence in Group I is significantly higher than that in Groups II and III, and the results clearly indicate that under the experimental conditions described the phenolic fraction of cigarette smoke condensate has considerable tumour-promoting activity but no carcinogenic activity.

DISCUSSION

Recent reports (Engelbreth-Holm and Ahlmann, 1957; Orris *et al.*, 1958; and Bock and Moore, 1959) have confirmed the findings of Wynder and his colleagues (Wynder, Graham, and Croninger, 1953; and see Wynder, 1959, for review); and the view is becoming generally accepted that cigarette smoke condensate is carcinogenic for mouse skin when applied at short intervals for long periods. The results of Experiments I and II in which the whole smoke condensate and its neutral fraction were tested in this way were unsatisfactory because of poor survival of mice: a few benign tumours arose in both experiments after 35 weeks of continuous treatment, and 2 malignant tumours at a much later date in the neutral fraction experiment.

Much attention has been given to the identification of the carcinogenic components in tobacco smoke condensate. Kennaway and Lindsey (1958) give a long list of individual substances which have been identified in smoke, and a short

list of 10 for which there is at present evidence of carcinogenicity. Most attention has been paid to the 3,4-benzpyrene content. Cooper, Lindsey and Waller (1954), Latarjet *et al.* (1956), and Bentley and Burgan (1958) all reported amounts of the order of 1 μg. of 3,4-benzpyrene in the smoke from 100 g. cigarettes. Wynder and Wright (1957) and Wynder and Hoffmann (1959) found slightly larger amounts than this, namely, about 4 μg. per 100 g. cigarettes. Cardon *et al.* (1956) on the other hand, estimated the benzpyrene, yield from 100 g. cigarettes at between 10 and 12 μg., but the calculations on which these higher estimates are based have been criticised by Bentley and Burgan (1958). Whichever estimate is correct it is very doubtful whether the amount of benzpyrene in smoke condensate is sufficient to account for the tumours seen in experiments with mice. Approximately 4 g. of condensate are produced by smoking 100 cigarettes (of 1 g. each), the amount varying according to the smoking conditions used. If we suppose that the higher figure given by Cardon *et al.* is correct, 4 g. of condensate would contain 12 μg. of 3,4-benzpyrene, and 40 mg. of condensate (the amount applied thrice weekly by Wynder and his colleagues) would contain approximately 0·1 μg. If, on the other hand, the figures given by Cooper, Lindsey and Waller, Latarjet *et al.*, and Bentley and Burgan is correct, the amount of 3, 4-benzpyrene per application would be $< 0{\cdot}01$ μg. Poel and his colleagues (see Poel, 1959) applied 0·38 μg. 3,4-benzpyrene to mice thrice weekly: two carcinomas (after 82 and 83 weeks respectively) and 9 papillomas (after an average of 69 weeks) arose in a group of 55 C57 mice. The same workers produced 5 papillomas (average induction time = 60 weeks), but no malignant tumours, in a similar group of mice painted thrice weekly with 0·15 μg. 3,4-benzpyrene. Even allowing for different experimental techniques and the strains of mice used, it seems most unlikely that the carcinogenic effect of smoke condensate observed by Wynder's group, and by others including ourselves, is due to 3, 4-benzpyrene alone.

Estimates of the amounts of the other carcinogens listed by Kennaway and Lindsey (1958) are either not available or highly conjectural. But, taking into account all the available data, and in particular the fact that several of the other carcinogens present are much weaker than 3,4-benzpyrene, it is unlikely that their carcinogenic effect combined with that of 3,4-benzpyrene could equal that of the whole smoke condensate. Dr. Wynder expressed the same view at a meeting held at the Chester Beatty Research Institute in July 1959. Of course it may be argued that since several of the substances listed by Kennaway and Lindsey have not been tested for carcinogenic activity, their possible contribution cannot be assessed. But to assume that their concentration and potency are sufficient to add appreciably to the carcinogenic effect of the benzpyrene and the other known carcinogens is surely inadmissible at this stage.

Turning to the possible role of incomplete carcinogens in tumour production by cigarette smoke condensate, the fact that a substance is carcinogenic when applied alone may be taken to indicate that it has both tumour-initiating and tumour-promoting properties. However, amounts of carcinogenic hydrocarbons too small to give detectable carcinogenic effect, e.g. 1 μg. 9-10-dimethyl-1, 2-benzanthracene (DMBA) (Klein, 1956) have demonstrable initiating action. Total doses of 3,4-benzpyrene of this order were undoubtedly applied in the course of the applications of smoke condensate by Wynder and others. Thus these amounts of condensate contained enough 3,4-benzpyrene for the initiation of the tumours

which arose, though certainly not enough for their production without the aid of a tumour promoter. Furthermore, in addition to the small amounts of 3,4-benzpyrene and other complete carcinogens, there may also be present in cigarette smoke condensate incomplete carcinogens of the type of which urethane and triethylene melamine are examples (Salaman and Roe, 1953; Roe and Salaman, 1955), i.e. substances with initiating but no promoting action. Little is known of the distribution of such substances, and their presence in tobacco smoke condensate has still to be demonstrated.

Thus on quantitative grounds alone we would expect to find tumour-promoting substances in cigarette smoke condensate. Experiment III reported here is a clear demonstration of tumour-promotion by the phenolic fraction, constituting about 15–20 per cent of the condensate. Commins and Lindsey (1956) estimated quantitatively some of the simpler phenols present in cigarette smoke, and recorded the total yields of 9 different phenolic compounds in micrograms per cigarette. The yield of phenol itself was 123 μg. per cigarette, quinol—83 μg., catechol—61 μg., and *ortho meta*, and *para*-cresol, together—80 μg. Resorcinol, 1-naphthol, and 2-naphthol were present in much lower quantities. The total yield of all 9 compounds amounted to 356 μg. per cigarette, which is equivalent to 0·36 g. per 1,000 cigarettes. The yields of phenol and *ortho*-cresol reported by Commins and Lindsey were close to those previously recorded by Rayburn, Harlan and Hanmer (1953).

In the experiments reported here the total yield of phenol fraction from 1,000 cigarettes was 4·8 g. (see Appendix). Thus the substances studied by Commins and Lindsey (1956) account for less than one tenth of the phenol fraction used in Experiment III, and the composition of the remainiug nine tenths is still not known.

In view of the known tumour-promoting activity of phenol and related compounds, (Boutwell, Rusch and Bosch, 1955; Salaman and Glendenning, 1957; Boutwell and Bosch, 1959) the result of Experiment III is perhaps not entirely unexpected. Quantitatively the tumour-promoting effect observed here was comparable to that for whole smoke condensate observed by Gellhorn (1958), i.e. the promoting activity of the phenolic fraction could have accounted for all the effect observed by Gellhorn. However differences in methods and strain of mice make such a quantitative comparison unwise.

If we assume that the carcinogenic effect of smoke condensate is due to the action of initiators (known or unknown) plus promoters (phenols and others), it is on the face of it difficult to explain why tests of the condensate for initiating action, by the simultaneous and/or subsequent application of croton oil, gave no tumours. Certainly promotion was more than adequate. Was enough initiator applied in these tests? In the experiments of Gwynn and Salaman (1956) a total of about 1·5 g. of smoke condensate was applied as an initiating dose. The benzpyrene content of this according to Cooper *et al.* (1954) and Bentley and Burgan (1958) would have been 0·3 μg., and according to Cardon *et al.* (1956) about 4 μg. This is of the order of the minimum initiating dose of DMBA reported by Klein (1956). Hamer and Woodhouse applied only about 0·2 g. of smoke condensate in their test for tumour-initiation, which would contain an amount of benzpyrene definitely below the detectable initiating dose. Thus it is probable that in the experiments specifically designed to show initiating action, too little initiator was applied.

We may sum up the present position with respect to tests on mouse skin by saying that :—

(1) the observed tumour producing action of cigarette smoke condensate is unlikely to be due to contained substances acting as complete carcinogens,

(2) it can be explained as due to the combined action of tumour initiators and promoters, and,

(3) since its effect can be increased by the addition of initiators but not of promoters, the relative weakness of the carcinogenic action of cigarette smoke condensate is due to the paucity of initiators.

There is at present no direct evidence for two-stage carcinogenesis in human bronchial epithelium. Assuming however that this mechanism operates here, as it has been shown to do in skin and some other tissues, present evidence suggests that smoking has stronger tumour-promoting than tumour-initiating effect.

Man, whether a smoker or not, is exposed to substances of proved tumour-initiating power in the polluted atmosphere, and in the products of some industrial processes and in some kinds of food. The correlation between smoking habits and lung tumour incidence may well be determined not primarily by the carcinogenic effect of tobacco smoke but by its predominantly tumour-promoting action on the bronchial epithelium.

SUMMARY

Strong tumour-promoting effect by a phenolic fraction of cigarette smoke condensate applied after a single tumour-initiating dose of 9,10-dimethyl-1,2-benzanthracene (DMBA) to the dorsal skin of " 101 " strain mice was observed : sixty-five benign and 2 malignant tumours arose on 30 treated mice during 40 weeks of treatment. The same dose of DMBA alone produced a negligible number of tumours, and the phenolic fraction alone produced none.

" 101 " Strain mice treated with whole smoke condensate only, or the neutral fraction of the condensate only, developed a few papillomas after 35 weeks. However, the survival of the mice was poor, and very few were alive at the time when malignant tumours might have been expected : only two were seen. One of the reasons for the poor survival was a renal disease, papillonephritis, previously recorded in strain A mice.

Reasons are given for believing that cigarette smoke is richer in tumour-promoting than in tumour-initiating substances. Phenolic compounds may be responsible for much of its promoting activity.

The implications of these findings, and their relevance to the induction of bronchial carcinoma in man, are briefly discussed.

We wish to express our gratitude to the Tobacco Manufacturers' Standing Committee for supplying us with tobacco smoke condensate and its fractions. We also wish to thank all the members of the technical staff of the department for their assistance. The expenses of the research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

APPENDIX: THE PRODUCTION OF CIGARETTE SMOKE CONDENSATE

(by J. G. Burgan, Research Laboratories, Tobacco Manufacturers' Standing Committee)

Smoking technique

Cigarettes, a mixture of brands popular in the United Kingdom, are smoked in batches of 1,000 using an automatic smoking machine of the type described by Iles and Sharman (1957), arranged to give a puff frequency of 4 per minute, a puff volume of 15 ml., and a puff duration of 2 seconds. Cigarettes are smoked to a stub length of 20 mm. The smoke condensate is collected in traps immersed in a dry ice/acetone mixture, one trap being attached to each smoking position. The type of cold trap used has been described by Burgan (1959).

Whole smoke condensate

For the production of whole smoke condensate the material collected in the cold traps is washed out with acetone. The solvent is then removed on the water-bath and the whole smoke so obtained is stored in the dark in sealed ampoules at −25° C.

Phenolic and neutral fractions

For the separation of smoke condensate into its main fractions the material from 1,000 cigarettes, collected in the cold traps, is dissolved in a mixture (1 : 3 v/v) of hydrochloric acid (2N) and ether (peroxide free), the ether used having been previously treated with sodium wire to remove fluorescent material. The aqueous acidic layer is separated and the ether layer washed further with portions of hydrochloric acid (2N, 4 × 100 ml.) to complete the removal of basic compounds.

The ether layer is then washed with portions of saturated sodium bicarbonate solution (1 × 200 ml., 4 × 100 ml.) to remove carboxylic acids, and with portions of 3 per cent potassium hydroxide solution (1 × 200 ml., 5 × 100 ml.) to remove any carboxylic acids remaining together with the phenols present.

The residual ether layer containing the neutral fraction of smoke condensate is dried over anhydrous magnesium sulphate and the solvent removed on the water-bath. The neutral fraction obtained is stored in the dark in sealed ampoules at −25°C.

The phenols are recovered from the combined potassium hydroxide washings. These are neutralized with hydrochloric acid (2N), excess sodium bicarbonate (solid) added, and the mixture saturated with sodium chloride and extracted with portions of ether (1 × 200 ml., 4 × 100 ml.). The combined ether washings, containing the phenols, are dried over anhydrous magnesium sulphate and the solvent removed on the water-bath. The phenols obtained are stored in the dark in sealed ampoules at −25° C.

The average amounts of whole smoke, phenols, and neutral fraction obtained from 1,000 cigarettes, using the smoking conditions described above, are respectively 29·5 g., 4·8 g. and 16·6 g.

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TUMOUR - INHIBITING ACTION OF 1,6 - Di - (2 - BROMOETHYL - AMINO) - 1,6 - DIDEOXY - D - MANNITOL DIHYDROBROMIDE [DBM (R13)]

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Received for publication September 11, 1959

NITROGEN mustard is at present the agent outstanding in the chemotherapy of malignant tumours. It furnishes the basis for most of the drugs prepared since Gilman and Philips (1946) synthesised the compound, by substituting a sulphide group for the amino group of mustard gas, and found it developed remissions of transplantable mouse lymphosarcoma. While the results it has yielded in Hodgkin's disease, lymphosarcoma, and chronic leukaemias, must not be undervalued (Goodman *et al.*, 1946 ; Jacobson *et al.*, 1946 ; Karnofsky, 1950), nitrogen mustard has proved to be of no value in the treatment of carcinomatous tumours.

Nitromin, prepared by Stahmann and Bergmann (1946) was the first nitrogen-mustard derivative to have a more marked growth-inhibiting effect on Yoshida sarcoma than nitrogen mustard itself, and to be of lesser toxicity (Ishidate, Kobayashi, Sakurai, Sato and Yoshida ; *cit.* Farber *et al.*, 1956). In Japan, Nitromin is being widely used in human therapy. Kimura and his co-workers (*cit.* Farber *et al.*, 1956) found that in addition to being of avail in cases of chronic leukaemia, it had a destructive effect on certain malignant tumours.

Another nitrogen-mustard derivative is Erysan (R_{48}), an aromatic chloroethylamine, first synthesised by Haddow and his co-workers (1948), which was shown to be highly effective on the Walker carcinosarcoma. Matthews (1950) reported its effectiveness in chronic myeloid and lymphoid leukaemia in humans, and Videbaek and Kaae (1954) in such cases of Hodgkin's disease as had failed to respond to X-ray treatment.

Everett, Roberts, and Ross (1953) synthesised N,N-di-(2-chloroethyl)-*p*-aminophenyl-butyric acid, an aromatic nitrogen-mustard derivative, which they labelled CB 1348. This compound was found considerably to inhibit the growth of Walker tumours and according to Haddow (1954), to have a beneficial effect on lymphoreticular tumours and haemoblastoses ; its depressant effect on bone marrow was less than that of nitrogen mustard and TEM, respectively.

Larionov (1957) reported good results obtained with Sarcolysin and Dopan. Simultaneously with, but independently of Larionov and co-workers (1955), Bergel and Stock (1954) also prepared sarcolysine dl-*p*-di(chloroethyl)-aminophenylalanine hydrochloride. It caused complete regression in two sarcomas of the rat and one of the mouse, and had a marked inhibitory effect on the growth of seven other mouse and rat tumours.

For some years Vargha has endeavoured to produce an agent of reduced toxicity, by linking nitrogen mustard with a sugar. Of the many compounds

he has prepared 1,6-di-(2-chloroethylamino)-1,6-dideoxy-D-mannitol dihydrochloride has proved to be of good effect. Having been investigated in animal experiments by Kellner and Németh (1956) and tested clinically by Sellei and Eckhardt (1958), it has been introduced in human therapy as Degranol (BCM).

MATERIALS AND METHODS

Twenty-three combinations of nitrogen mustard with sugar, prepared by Vargha in the course of the last two years, were tested for their respective toxicity, and studied in transplantable animal tumours for their growth-inhibiting action. Of these, one compound, marked R_{13}, the structural formula of which may be represented as 1,6-di-(2-bromoethylamino)-1,6-dideoxy-D-mannitol dihydrobromide (DBM), was found to be the most promising (Vargha and Horváth, 1959; Baló, Kendrey, Juhász, Besznyák, 1959). This is a stable compound readily soluble in water, of which 23 mg./kg. of body weight is the half-lethal dose (LD_{50}) for the mouse, and 22 mg./kg. that for the rat.

Using Wistar strain rats and white mice of our own breed, experimentation in normal intact animals and animals with transplanted tumours showed the daily therapeutic dose level to vary between 1·5 and 3 mg./kg. for the tumour-bearing rat, and 3 and 5 mg./kg. for the tumour-bearing mouse. In testing the compound against a spectrum of rat and mouse tumours, treatment, usually with daily intraperitoneal injections, was begun when the tumours were from a pea to a bean in size. A prolonged treatment not involving considerable loss of weight was aimed at. After treatment varying in time with the strain of tumour, the animals were killed, the tumours removed and measured, and the percentage growth–inhibition was determined on the basis of the following formula:

$$\text{Inhibition } \% = \frac{\text{Average weight of tumours in controls} - \text{Average weight of tumours in experimentals} \times 100}{\text{Average weight of tumours in controls}}$$

In some experiments survival time was used as a measure of benefit from the drug. In testing DBM against tumours in ascitic form, treatment was begun either one or two days after inoculation of the ascites, or on the first signs of ascites formation in the inoculated animal, and the degree of the inhibitory effect was graded according to the difference in body weight between the treated and the control animals.

The drug was subjected to detailed examinations for its effect on the blood picture, the bone marrow, and the parenchymatous organs.

In most cases the effectiveness of DBM was compared with that of Degranol (BCM).

RESULTS

Effect of DBM (R_{13}) on transplantable tumours

1. The growth-inhibiting action on rat tumours was studied in a set of experiments using Guérin carcinoma, Benevolenskaya sarcoma, and a subcutaneous form of Yoshida sarcoma. In agreement with Druckrey and co-workers (1958), we found the latter was just as useful as Yoshida ascites sarcoma in testing chemotherapeutic agents: rats inoculated with the tumour in ascitic form survived

for an average 8 to 10 days, with only 1 to 3 ml. of ascitic fluid forming; subcutaneously inoculated rats survived for 12 to 18 days.

The growth-inhibiting effect of DBM on the rat tumours involved in our experiments is illustrated in Table I and Fig. 1 and 2.

Using Guérin tumour, DBM was tested for its effect upon survival time in 39 Wistar strain rats treated in three groups with an intraperitoneal daily dose of 0·4, 1·0, and 2·0 mg./kg. of body weight, respectively; another 39 rats of the same strain were left untreated to serve as controls; an additional 24 rats bearing Guérin tumours were given from 4 to 12 mg. daily doses of Degranol per kg. of body weight. Treatment was continued until the death of the last experimental animal. In relation to the untreated controls, DBM was found to have prolonged the survival time by from 8 to 15, and BCM by from 2 to 7 days.

2. For its effect on transplantable solid mouse tumours the compound was studied on subcutaneously inoculated Ehrlich carcinoma and S-37 and S-180 (Crocker) sarcoma. The results obtained are summarized in Table II.

3. For its growth-inhibiting action in mouse tumours in ascitic form, DBM was tested in Ehrlich carcinoma and Amytal sarcoma. The latter originates from this Institute (Juhász, Baló and Kendrey, 1955); it is transplanted in every 10 to 12 days, during which time 8 to 10 ml. of ascitic fluid develop in each animal; the mice generally survive transplantation by from 14 to 18 days. To every ml. of ascitic fluid drawn from them, 0·25 mg. of DBM and Degranol, respectively, were added dissolved in 1 ml. saline under sterile conditions and the mixture was kept in the thermostat at 37° C. for 45 minutes. Two groups of 10 white mice each were then inoculated with an equal amount of one and the other of these mixtures, respectively, while in a third group of 10 controls each animal was inoculated with a corresponding amount of a mixture of ascitic fluid and sterile physiological NaCl incubated likewise at 37° C. for 45 minutes. All the animals were kept under observation for 11 days with a view to finding out to what extent under the identical conditions equal concentrations of the two drugs were capable of depressing takes and inhibiting tumour growth. The results of daily checks on the changes of body weight showed that while the average over-all gain was 7·10 g. in the control group, it amounted to 3·23 g. in the group treated with DBM, and to 5·74 g. in that treated with Degranol; in other words, an inhibitory effect of 44 per cent. was observed upon the action of DBM, and of 19·2 per cent. upon that of Degranol.

Using Amytal ascites sarcoma, another experiment was carried out to determine the influence of DBM and Degranol, respectively, on the survival time of mice. Fig. 3 shows that while seven consecutive daily doses of 4·85 mg. of DBM per kg. of body weight resulted in prolongation of survival time, no appreciable prolongation followed the administration of the same number of 21 mg. doses of Degranol.

EXPLANATION OF PLATE

FIG. 1.—Showing 92 per cent growth inhibition on Guérin carcinoma after 19 doses of 2·6 mg./kg. of DBM (R_{13}).

FIG. 2.—Showing 93 per cent and 84 per cent growth inhibition on subcutaneously inoculated Yoshida sarcoma after 9 doses of 1·75 mg./kg. of DBM (R_{13}) and 10 mg./kg. of Degranol (BCM), respectively.

FIG. 5.—Showing inhibition of development of Ehrlich ascites carcinoma by 7 daily doses of 4·85 mg./kg. of DBM (R_{13}).

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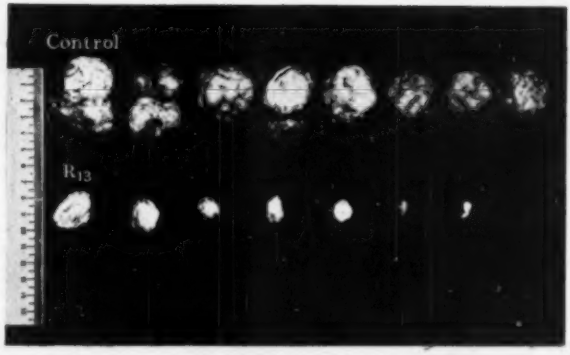

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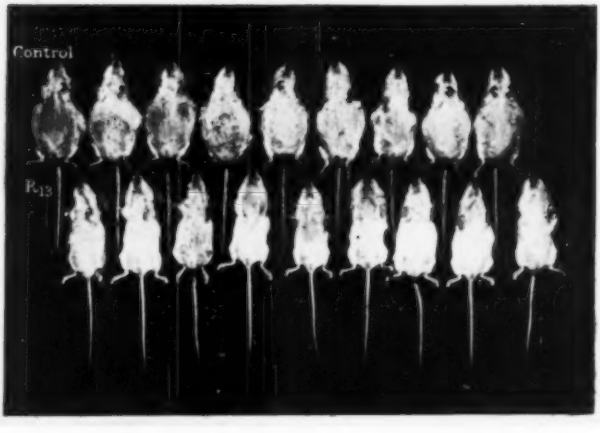

TABLE I.—*Degree of Inhibition of the Growth of Certain Rat Tumours by DBM (R_{13}) and Degranol (BCM)*

Compounds tested	Tumours used	Number of rats		Daily dose (mg./kg.)	Duration of treatment (in days)	Number of treatments	Average weight of tumours		Loss of weight (in %)	Inhibition of growth (in %)
		Treated	Control				Treated (g.)	Control (g.)		
DBM	Guérin rat carcinoma	15	15	2	49	24	7·80	70·00	—	89
DBM	,, ,, ,,	10	10	2·6	28	19	1·36	17·52	16	92
DBM	,, ,, ,,	11	11	1·96	17	12	2·10	5·82	—	64
Degranol		11	11	12·00	17	12	2·70	5·82	—	34
DBM	Yoshida, rat sarcoma subcutaneous form	10	10	1·85	13	9	0·24	8·40	7	97
Degranol		10	10	15·00	13	9	0·17	8·40	12	98
DBM	Ditto	8	8	1·75	14	8	1·22	16·77	0	93
Degranol		8	8	10·00	14	8	2·53	16·77	6	84
DBM	Benevolenskaya rat sarcoma	11	11	2·25	24	10	8·08	25·65	0	68
Degranol		11	11	12·75	24	10	8·86	25·65	3	66

TABLE II.—*Degree of Inhibition of the Growth of Certain Mouse Tumours by DBM (R_{13}) and Degranol (BCM)*

Compounds tested	Tumours used	Number of mice		Daily dose (mg./kg.)	Duration of treatment (in days)	Number of treatments	Average weight of tumours		Inhibition of growth (in %)
		Treated	Control				Treated (g.)	Control (g.)	
DBM	Ehrlich mouse carcinoma	8	8	3·00	12	8	1·33	2·18	34
Degranol		8	8	15·00	12	8	1·73	2·18	21
DBM	S-37 sarcoma	10	10	4·85	15	10	0·98	2·10	53
Degranol		10	10	14·00	15	10	1·71	2·10	28
DBM	S-180 (Crocker) sarcoma	12	12	4·85	12	9	1·55	3·61	57
Degranol		12	12	21·00	12	9	1·67	3·61	53

On the growth of Ehrlich ascites carcinoma, 11 consecutive daily doses of 6·75 mg. of DBM per kg. of body weight were found to have a marked inhibitory effect, while the same number of 14 mg. doses of Degranol failed to inhibit essentially the growth of this tumour (Fig. 4).

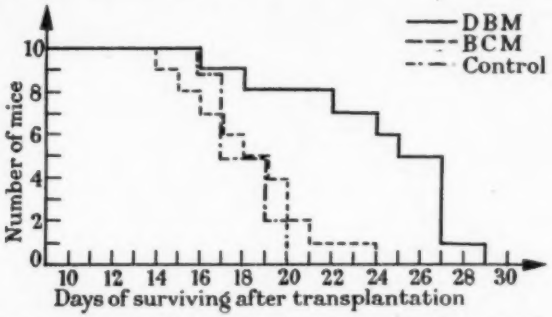

FIG. 3.—Showing increase of survival time of mice inoculated with Amytal ascites sarcoma treated with DBM (R_{13}) or Degranol (BCM).

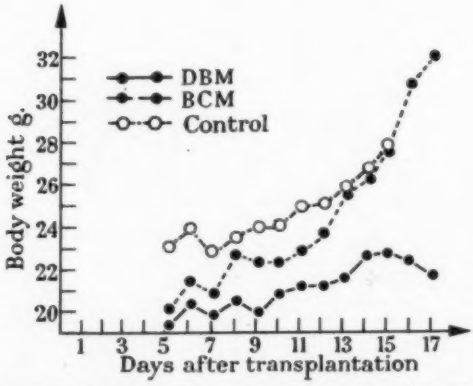

FIG. 4.—Showing increase of weight of mice bearing Ehrlich ascites carcinoma following treatment with DBM (R_{13}) or Degranol (BCM).

In another experiment, in which animals bearing Ehrlich ascites tumours were treated during 9 days on each of seven occasions with 4·85 mg. of DBM per kg. of body weight, no ascites developed at all (Fig. 5). Of Degranol, the same number of 21 mg. doses was required to achieve the identical effect.

Effect of DBM on blood picture, bone marrow, and parenchymatous organs

For its effect on peripheral blood DBM was studied in 16 rats of the Wistar strain, each weighing 150 g. After their complete blood pictures had been prepared, some normal animals were injected intraperitoneally on one occasion with a simple, others with a threefold, yet others with a tenfold medium therapeutic dose (the

simple *dosis therapeutica* being 1·75 mg./kg. of body weight). Twenty-four hours later, there was no change in the number of erythrocytes at any dose levels; the simple therapeutic dose was followed by minimal lymphopenia ; the threefold dose gave rise to significant lymphopenia and monocytopenia and relative leukocytosis, though the white cell count remained normal. The tenfold dose caused appreciable leukopenia, the lymphocytes disappeared almost completely from the blood, the plasma of the retained ones becoming vacuolated and, in addition, a marked decrease in the number of monocytes and a relative leukocytosis were observed (Table III).

TABLE III.—*Effect of a Single Intraperitoneal Dose of DBM Upon the Blood Cells*

Dose (mg./kg.)		Number of erythrocytes (10^6/c.mm.)	Number of leukocytes (10^3/c.mm.)	Poly.	Ly.	Mo.	Eo.
1·75	Before treatment	6·4	8·2	42	55	2	1
	After treatment	7·8	9·2	43	53	4	0
5·25	Before treatment	8·5	10·7	36	59	3	2
	After treatment	7·9	9·1	62	37	0	1
17·5	Before treatment	8·5	9·2	25	72	1	2
	After treatment	8·4	6·6	92	6	0	2

In animals given the medium therapeutic dose for 15 consecutive days, there was no change in the red cell count, but the white cells fell to 5000 per c.mm. from the normal of about 9000 per c.mm. generally counted in the control animals ; in the number of lymphocytes a substantial decrease was observed ; relative leukocytosis presented itself, associated with eosinophilia (Table IV).

TABLE IV.—*Effect of Serial Intraperitoneal Treatment* (15 *Times*) *with DBM Upon the Blood Cells of Rats*

Daily dose (mg./kg.)		Erythrocytes (10^6/c.mm.)	Leukocytes (10^3/c.mm.)	Poly.	Ly.	Mo.	Eo.
1·75	Before treatment	8·2	9·0	34	62	2	2
	After treatment	8·3	5·0	45	46	3	6

For its action on bone marrow the compound was tested in normal intact Wistar strain rats which in 45 days, had been treated on 34 occasions with 1·75 mg. of DBM per kg. of body weight by the intraperitoneal route. This treatment was followed by a slight decrease in the number of myeloid elements. In some individual cases the haemopoietic colonies were seen to have ceded their place to fatty bone marrow. The blood pictures of these animals revealed marked lymphopenia, relative leukocytosis, and moderate eosinophilia. No pathological changes were encountered in the myocardium, the lung, the kidney, and the adrenal gland, but the spleen was found to have decreased in size, and the lymph follicles were less than normal in number. Slight pulpar fibrosis was seen. In the liver parenchymatous degeneration was observed, but no fatty degeneration or cellular necrosis.

DISCUSSION

On the evidence of the results of our toxicity tests, the efficient but not toxic dose of DBM varies between 1·5 and 3·0 mg. per kg. of body weight for the tumour-

bearing rat, and between 3·0 and 5·0 mg. for the tumour-bearing mouse. At these dose levels, the animals will stand treatment for 10 to 12 consecutive days without losing more than 6 to 12 per cent. in weight. The chemotherapeutic index for DBM being

$$\frac{\text{MTD}}{\text{MED}} = 25,$$

it comes to fall between that for Degranol (50) and nitrogen mustard (10).

From our experimental findings the conviction accrues that DBM is a drug, which exerts a marked inhibitory effect on tumour cells. Undoubtedly, it was the most effective against Yoshida sarcoma, a tumour responding as a rule fairly well to efficient chemotherapeutic agents. Equally remarkable was the effect it appeared to have on Guérin cancer, the more so as malignant animal tumours (Guérin and Ehrlich tumours) are conspicuous for the difficulty in influencing their growth by means of therapeutic drugs. Not a single complete regression was observed in animals inoculated with these tumours, unlike in cases of subcutaneously implanted Yoshida sarcoma. Histologically, the small tumours found in a few of our animals inoculated with Yoshida sarcoma and treated with DBM could no longer be regarded as real tumours.

In animals bearing Guérin or Ehrlich carcinoma, we on a few occasions succeeded in maintaining for some considerable period of time a state in which there was no perceptible tumour growth, and even several remissions took place. If in such a state treatment was continued, the animals died after a time, displaying toxic symptoms ; if it was discontinued, the tumour started to grow again but could not be depressed once more, not even by means of larger doses.

With the histological changes in tumours following treatment with DBM, we propose to deal elsewhere.

SUMMARY

Twenty-three compounds prepared by linking nitrogen mustard with various sugars were tested on various transplantable tumours for their capacity to inhibit growth. The one designated as DBM (R_{13}), the structural formula of which may be represented as 1,6-di-(2-bromoethylamino)-1,6-dideoxy-D-mannitol dihydrobromide, was found to have a marked inhibitory effect on Guérin rat carcinoma, subcutaneous form of Yoshida rat sarcoma, Benevolenskaya rat sarcoma, the subcutaneous and ascitic forms of Ehrlich mouse carcinoma, Crocker and S-37 mouse sarcoma, and Amytal ascites sarcoma in the mouse. The drug is much less toxic than nitrogen mustard and depresses tumour growth without causing considerable loss of weight or toxic side effects. It is not without effect on peripheral blood and the lymphatic organs. Following treatment with therapeutic doses for some longer period of time, there is a reduction first in the lymphocyte and then in the leukocyte count, the spleen diminishes in size, the lymph follicles in it grow less in number, and fibrosis of the pulp is seen. Animal experiments have shown this agent to be more effective than Degranol.

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AN OSTEOGENIC SARCOMA IN THE FOWL

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Received for publication October 27, 1959

SPONTANEOUS primary bone tumours are so rare in the fowl that the occurrence of an osteogenic sarcoma is of sufficient intrinsic interest to warrant report, particularly since Feldman and Olson (1952) state that the rarity of these neoplasms has precluded adequate study.

Osteomata have been observed in the fowl by Fujinami (1930) and Heim (1931). Kaupp (1933) described a tumour which he termed a sarco-chondro-osteoma. Avian osteoclastomata have been reported by Baker (1928) and Campbell (1947). Tytler (1913) described in detail a transplantable osteo-chondro-sarcoma in fowls. Eber and Malke (1932) mentioned the occurrence of an osteosarcoma among their large collection of avian neoplasms. Reis and Nobrega (undated) state that in birds they have seen five osteogenic sarcomata, three of which presented the appearance of osteo-chondro-fibromata. Neither Eber and Malke nor Reis and Nobrega described these tumours in any detail.

This communication describes the finding of a malignant osteogenic sarcoma, which had arisen spontaneously at the proximal extremity of the right tibia, and had metastasised to the right kidney and to both lungs.

Case history

A Brown Leghorn female (A.3466) was found to be lame when over seven years old. She was one of a group of normal hens kept at the Poultry Research Centre under ordinary semi-intensive conditions as controls to a long-term environmental experiment, briefly reported on by Greenwood (1958) and Wilson (1958). A hard swelling had developed in the region of the right femoro-tibial articulation. Movement of this joint was somewhat restricted but the degree of lameness was mild. On palpation the swelling was found to be hard, apparently painless and firmly adhering to the bone. A radiograph (Fig. 1) showed a tumour involving the proximal extremity of the tibia; it affected neither the femur nor, apparently, the joint cavity. The tumour was considerably calcified and the " explosive " pattern on the X-ray was very suggestive of that seen in human osteosarcomata. The plasma alkaline phosphatase activity of this bird was 360 units (mg. of 4-nitrophenol liberated by 100 ml. of plasma at pH 10, during 60 minutes at 37° C.).

Post mortem findings

A large tumour, about 3 cm. in diameter and of very firm consistency was situated near the proximal extremity of the tibia. The femoro-tibial joint itself and the distal extremity of the femur were unaffected. No other bones of the body were found to be involved. Numerous very firm nodular metastases of up to 5 mm. in diameter were scattered throughout the substance of the right

kidney. Both lungs carried similar tumours of varying size. The other organs, including the left kidney, showed no macroscopic abnormality.

Transmission experiment

No tumours resulted from injections of saline tumour suspension in day-old chicks. Similar material grew on the chorio-allantoic membrane of 3 out of 6 inoculated 10-day chick embryos, but further passage failed.

Histo-pathology

Material from the primary growth on the tibia was fixed in 10 per cent formal-saline and decalcified. Blocks of other tissues and organs were fixed in Susa, embedded in paraffin and sectioned without previous decalcification.

The tibial tumour showed a structure typical of osteogenic sarcomata, with large areas of osteoid and calcified bone (Fig. 2) and, particularly at the periphery, areas of more or less undifferentiated spindle-shaped osteoblasts (Fig. 3). The osteoid and calcified bone was arranged in irregular trabeculae which were bordered by chains or sometimes large groups of pleomorphic, somewhat spindle-shaped osteoblasts, which frequently bore numerous processes (Fig. 4). In section the free " edge " of the tumour showed sheets of closely knit undifferentiated cells, exhibiting marked infiltrative growth into the surrounding muscle tissue (Fig. 5). Although multinucleated giant cells may have been present they were by no means obvious, even in the calcified portions of the neoplasm. In the undifferentiated peripheral portion mitotic figures, although present, were not numerous ; the nucleoli of the osteoblasts in these areas were, however, large and very prominent (Fig. 3). The tumour was well vascularised and there was no evidence of necrosis.

The histological appearance of the metastases was essentially similar to that of the primary tumour. In the lungs, as well as in the kidney, these secondary growths appeared to be fairly well circumscribed and more or less spherical ; they were not encapsulated and, in fact, manifested apparently rapid infiltrative growth into the tissues of the affected organs.

In the centre of these metastases, well differentiated and partially calcified osteoid was present, particularly in the lungs. This was irregularly interspersed by groups of readily recognisable osteoblasts. The peripheral infiltrating zone had a much more anaplastic character, the tumour cells exhibiting great pleomorphism and numerous mitotic figures (Fig. 6).

The apparently rapid infiltrative growth of the metastatic tumour cells in the kidney is illustrated in Fig. 6, where atrophic, but in no way degenerate, renal tubules and glomeruli were seen embedded in tumour tissue. In the lungs the appearance was essentially similar in that the alveoli tended to become filled with tumour cells, and in the peripheral portion at any rate, they retained a suggestion of lobulated structure. Areas of necrosis were not observed in the metastatic growths.

DISCUSSION

There can be little doubt that this tumour is an osteogenic sarcoma. Its histological and radiological characteristics are virtually identical with those of human osteosarcomata. According to the classification of Coventry and Dahlin

(1957) the tumour resembles a human osteoblastic osteogenic sarcoma on the basis of the predominance of more or less anaplastic osteoblasts and the presence of a variable but definite osteoid component. Calcification was seen not only in the primary tumour but also to some extent in the metastases. According to Dahlin (1957) multinucleated giant cells are rarely seen in osteogenic sarcomata ; in the present case such cells were not observed.

It is unfortunately impossible to compare the histology of the present tumour with that of other osteosarcomata occurring in fowls. The only other cases which have been found in the literature were reported by Eber and Malke (1932) and Reis and Nobrega (undated), neither of whom give any histological details.

Authorities agree that the most common sites for osteogenic sarcomata in man are the distal extremity of the femur and the proximal end of the tibia (Christensen, 1925 ; Geschickter and Copeland, 1949 ; Dahlin, 1957 ; Amromin, 1959). While in the avian case of Eber and Malke the primary tumour was situated on the sternum, in the present case it was found to be at the proximal extremity of the right tibia. Reis and Nobrega make no mention of the location of their tumours.

Two-thirds of the human osteogenic sarcomata occur at an age of between 10 and 30 years ; very young children and old people are seldom affected (Willis, 1953). If a true comparison were at all possible, the present case would be found to fall very much outside this range ; at the time when the tumour was diagnosed the hen was over seven years old, an advanced age for a domestic fowl. It is possible that one reason why this counterpart of the most common primary malignant human bone tumour is so rarely observed in fowls, is that they are not kept commercially beyond a maximum age of about 3–4 years.

The plasma alkaline phosphatase activity in this present case was 360 units ; the range for non-laying, non-moulting, normal hens lies between 25 and 120 units (Bell, 1960). High alkaline phosphatase activity is also characteristic of human osteogenic sarcomata (Willis, 1953). The alkaline phosphatase of the plasma is believed to originate largely if not entirely, in the osteoblasts ; the enzymic activity of the plasma may therefore be considered to reflect the amount of osteoblastic activity in the bone. In birds suffering from " cage layer fatigue ", which involves a bone dystrophy, high values for plasma alkaline phosphatase (300–600 units) have been invariably found (Bell, Siller and Campbell, 1959).

EXPLANATION OF PLATES

FIG. 1.—Radiograph of the right leg, showing a large calcified tumour at the proximal end of the tibia. The femur and the femoro-tibial joint were not affected. Note the partially calcified tendons.

FIG. 2.—Section of the primary tumour shown in Fig. 1. Note the high degree of differentiation with the formation of osteoid and calcified bone. There are large masses of mature osteoblasts, but no osteoclasts. H. & E. ×95.

FIG. 3.—Illustrates the more anaplastic character of the osteoblasts at the periphery of the primary tumour. H. & E. ×385.

FIG. 4.—Calcified and non-calcified osteoid of the tibial neoplasm, showing various degrees of maturity in the osteoblasts ; some bear very pronounced processes. H. & E. ×385.

FIG. 5.—Infiltrative growth of the primary tumour. Note the masses of anaplastic osteoblasts invading the neighbouring muscle tissue. H. & E. ×95.

FIG. 6.—Metastasis in the right kidney, showing atrophied tubules and glomerulus and also mitotic figures. H. & E. ×385.

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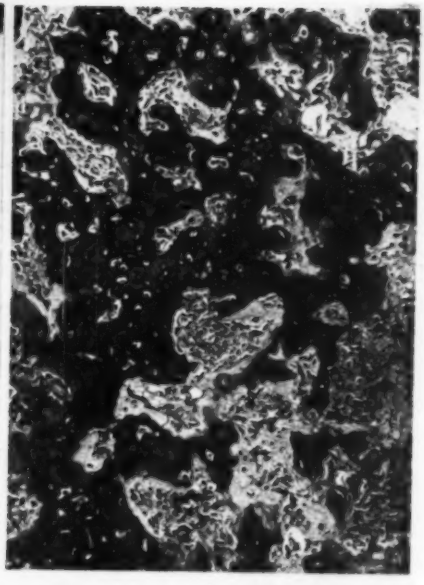

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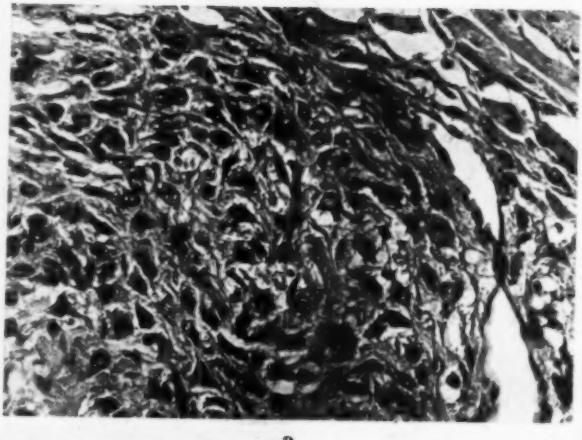

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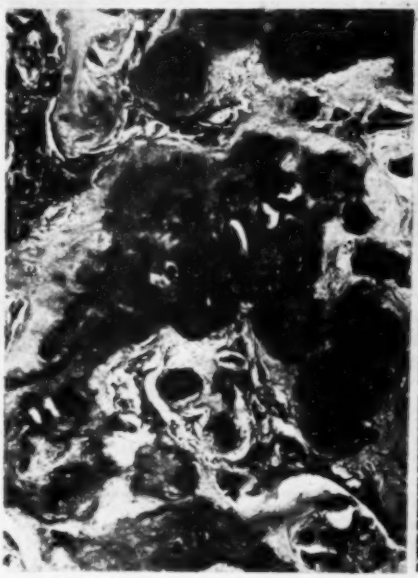

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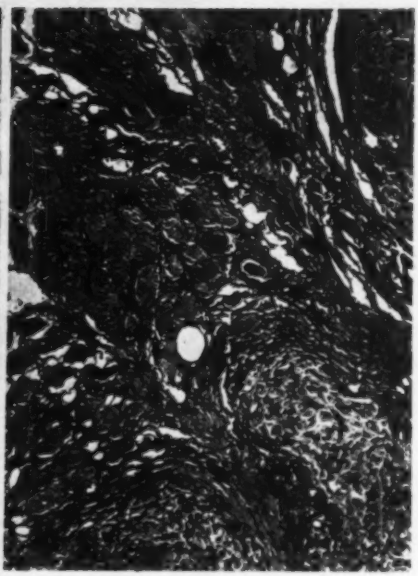

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In view of the fact that some bone tumours in the fowl, such as the osteoma of Fujinami (1930), the osteo-chondro-sarcoma of Tytler (1913) and the osteoid sarcoma of Pikowski and Doljanski (1946), are transplantable, it is noteworthy that the transplantation experiments failed in the present case.

Metastases to the lung occur very frequently in man. Willis (1953) assesses the incidence of secondary growths in the lungs as high as 75 per cent. Spread is generally by the haematogenous route and lymphatic dissemination is rare. In the present case both lungs carried metastases. A very interesting phenomenon is the confinement of renal metastases to that organ situated isolaterally to the affected leg. Birds possess a renal portal circulation (Spanner, 1924–25), whereby venous blood from the leg is filtered through the capillary bed of the isolateral kidney. The fact that heavy metastases were found in the right kidney, and none in the left, supports the conclusive work of Sperber (1948) on the existence of a functional renal portal system. This would provide an immediate haematogenous pathway between the primary tumour and the capillary bed in the kidney. Despite Sperber's experimental results and his carefully summarised evidence for and against the existence of a functional renal-portal system there are some (e.g. Barth, 1949) who still question its existence in the adult fowl.

SUMMARY

A case of spontaneous osteogenic sarcoma is described in a seven-year-old female fowl. The primary tumour developed on the proximal extremity of the right tibia and there were metastases in the right kidney and both lungs. The radiological and histological examination of this neoplasm showed the characteristic picture of human osteogenic sarcomata. A high plasma alkaline phosphatase activity was observed in this fowl. Transmission experiments were negative.

The occurrence of the isolateral renal metastases is interpreted as additional evidence for the existence in adult fowls of a functional renal portal system.

The author thanks Dr. D. J. Bell of the Biochemical Section for the determination of the alkaline phosphatase activity; he also thanks Dr. J. G. Carr of the British Empire Cancer Campaign Unit for carrying out the transplantation experiments.

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ON A HORMONE-DEPENDENT SPONTANEOUS, MALIGNANT ASCITES TUMOUR IN RATS

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Received for publication September 12, 1959

In recent years much interest has been focused on ascites tumours. They were originally obtained from primary tumours in rats and mice that had been treated in some way or other. The ascites tumours were then transplanted in series through a large number of animals. One of the types of tumours developing in rats was the Yoshida sarcoma. Cells from this tumour proliferate in the abdominal cavity and sometimes infiltrate surrounding tissues with formation of solid, massive growth, which soon kills the host. Thick ascites, a suspension of tumour cells, forms in the peritoneal cavity. This material can be injected into the abdominal cavity of normal rats and the tumour thus carried in series. Other similar rat sarcomas are Takeda-, MTK- and Hirosaki sarcoma (Yoshida, 1953).

A search of the literature failed to reveal any report on the spontaneous occurrence of primary ascites tumours in untreated rats.

This paper is concerned with a spontaneous ascites sarcoma that was found to be fairly common in a closed rat colony. The tumour was successfully transplanted by intraperitoneal, subcutaneous and intravenous injection. It was made the subject of chromosomal studies and proved to be dependent on sex hormones.

In the beginning the tumour appeared to be more common in males than in females. In an attempt to reveal any relationship between sex hormones and the development of the tumour, groups of intact male and female rats, castrated males, and spayed females were compared. One group of spayed rats received oestrogen. In addition, the frequency of the tumour was studied in parabiotic rats, of which one partner was an intact and the other a spayed female. Gonadotrophins from the spayed female pass over to the intact female and stimulate its ovaries (Zeckwer, 1946). In the intact female partner in parabiosis the oestrogen production therefore is fairly constant and relatively high. The oestrogens are not transferred to the spayed partner.

MATERIAL AND METHODS

The observations were made in animals of one and the same closed colony that has been maintained, without brother-sister mating, at the Department of Obstetrics and Gynaecology in Lund. All the animals have been brought up in cages of approximately equal size and on the same diet consisting of grain, milk and kitchen refuse.

Some of the animals—males and females—were castrated or spayed at 3 weeks of age, others were left intact. Some of the spayed and intact female rats of

the same litter were joined in parabiosis, coelio-anastomosis, at 4 weeks of age. Some of the spayed females received oestrogens in oil solution (0·25 ml. i.m. = 1·25 mg. oestradiolbenzoate/week) for 11 months.

The experimental material is given in Table I.

TABLE I.—*Number of Experimental Animals Above 9 Months of Age*

(The first tumour was observed at this age)

Intact males	45
Castrated males	50
Intact females	134
Spayed females	102
,, ,, treated with oestrogen	48
,, ,, in parabiosis with intact animals	24
Intact females in parabiosis with spayed	24
Total	427

Most of the animals were allowed to live until they died spontaneously. Some were killed to secure fresh material for histological examination. All the animals were examined *post mortem*.

RESULTS

The tumour was never observed in intact or spayed females before the animals were 9 months of age. In males they did not appear until still later. The oldest animal in which a tumour of this type developed was a spayed female, 20 months old. The frequency and age distribution of the animals killed by the tumour during the period of observation are given in Fig. 1.

Statistical analysis showed that the material permitted no definite conclusions regarding the frequency of the tumour in the different groups of animals until the animals were more than 12 months old. After this age limit the frequency of tumours was higher in spayed females compared with intact females and spayed rats treated with oestrogen. The difference between the frequency expected and that observed was 4 times the mean error.

No difference in frequency of the tumour was found between spayed females and spayed females that had lived in parabiosis with intact females. Parabiosis between spayed females and intact females produced no statistically significant inhibition of tumours in the intact females.

Neither was any difference found between the frequency of tumours in males and females or between intact and castrated males.

As a rule, the animals died within 2–3 weeks after the appearance of demonstrable symptoms. They lost weight rapidly and became severely anaemic. The abdomen swelled severely with ascites and tumour (Fig. 2). At autopsy the peritoneal cavity was filled with cloudy, usually slightly blood-stained fluid. Sometimes it was also milky or almost water-clear. The ascitic fluid contained a large number of tumour cells loosely dispersed or in small clumps. The nuclei of the cells varied in size and shape. The appearance of the tumour cells is so characteristic that the cells are readily distinguished from blood cells (Fig. 3). Chromosomal analysis was performed at the Cancer Chromosome Laboratory in Lund (Hansen-Melander and Melander, unpublished). Excellent material for chromosomal studies is also obtained from the soft solid tumours.

The solid tumours, usually very widespread, were soft and richly vascularised. The tumour showed a distinct tendency to haemorrhage and necrosis, sometimes with secondary infection. The tumour tissue grew in the omentum or on the peritoneum and in large confluent infiltrates in the ileocolic mesentery or retroperitoneally (Fig. 4). The cut surface of the tumour was solid and white or slightly pink. The mediastinum often showed metastases, usually adherent to and infiltrating the thymus. Then the pleural cavities contained fluid of the same type as the ascites in the abdomen. Metastatic growth to the lymph nodes

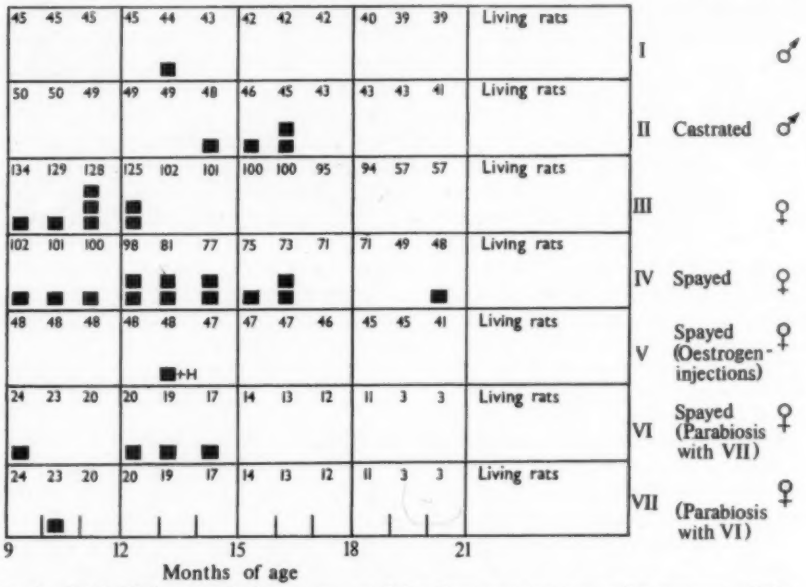

FIG. 1.—Each filled square indicates one rat dying from spontaneous ascites tumour. Only one animal, in Group V, had a co-existent tumour elsewhere (in hypophysis). The numerals designate survivals in various age classes.

in the groins, axillae and neck was observed. On the other hand, the liver, lungs, kidneys and spleen appeared not to be involved, not even microscopically. The largest tumours were situated in the ileocaecal mesentery, which appeared to be the original site of the tumour, possibly in the lymphatics which there run parallel to the ascending colon and extend cranially along the vasa mesenterica superior. The outer layers of the wall of the colon were seldom infiltrated, and the intestinal mucosa was intact.

The tumours, both the large retroperitoneal infiltrates and the various metastases, showed the same microscopic picture (Fig. 5) with diffuse growth of anaplastic tumour cells, cellular and nuclear polymorphism. The cytoplasm was relatively sparse and acidophilic. Cells with large, lobated nuclei were common. The nuclei often lay eccentrically in the cells. Mitoses were extremely common. The stroma was scanty and consisted of a few fine fibrils or trabeculae, rich in

small blood vessels. Haemorrhages, necroses and infiltration with lymphocytes and leucocytes were common. The tumour may possibly be regarded as a polymorpho-cellular reticulum-cell sarcoma.

The rats used as hosts in the transplantation experiments belonged to the same colony and were, as a rule, 1–2 months old. After intraperitoneal inoculation of 0·5 c.c. ascites or a small piece of homogenised tumour suspended in saline the ascites sarcoma grew rapidly in some of the new hosts. After a few passages successful inoculation usually killed the animals within two weeks. During the last few days before the animals died large amounts of ascitic fluid accumulated in the abdomen, which was markedly distended. In addition, solid growths always occurred in one or more of the sites where the primary tumours had been found. Inoculation was followed by tumour growth in males and females, gonadectomised or intact, and independently of the fact whether they had received ascitic fluid or homogenised solid tumour.

For some unknown reason in some of the animals the primary tumour failed to take. After rather many successful passages the tumour sometimes suddenly failed to take. This might have been due at least in part to genetical heterogeneity of the strain and chromosomal changes in the tumour during its passages. Of 15 primary tumours takes were obtained with 7. One tumour was carried through 18 generations. After subcutaneous inoculation of ascites solid tumours developed at the site of inoculation. After intravenous injection (into the tail vein) of ascites or homogenised solid tumour an extensive tumour growth was observed in the same sites as after intraperitoneal inoculation.

DISCUSSION

The growth of ascites cancer cells in heterologous hosts was found by Ahlström and Ising (1955, 1956) to vary with sex. On transplantation of mouse ascites carcinoma to hamsters growth was more abundant and the yield of tumour cells larger in females than in males.

The tumour described here, probably a reticulum-cell sarcoma, growing also in ascitic form, and probably of high degree of malignancy (developed early in life of the animals and killed them rapidly) was found to be more common after oophorectomy and to be inhibited by administration of oestradiol. Tumours in the lymphatic tissue (lymphosarcoma) in mice, on the other hand, occur more frequently in animals treated with oestrogens (Shimkin, 1957).

SUMMARY

A spontaneous ascites tumour probably a reticulum-cell sarcoma, that developed in a closed rat colony is described. All of the animals with such a tumour died between 9 and 20 months of age.

EXPLANATION OF PLATE

FIG. 2.—Rat with spontaneous malignant abdominal tumour producing ascites. One week before death.

FIG. 3.—Tumour cells in ascitic fluid. Papanicolaou. × 600.

FIG. 4.—Extensive growth of partly haemorrhagic tumour in mesentery and omentum (compare Fig. 2).

FIG. 5.—Tumour growth in omentum. 6 μ section. Haematoxylin and eosin. × 600.

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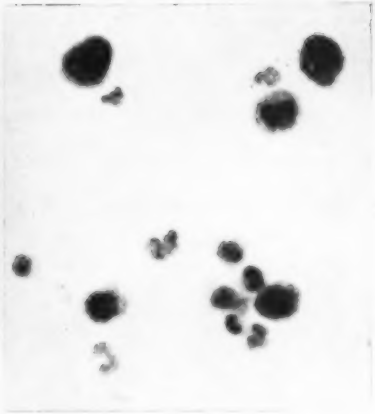

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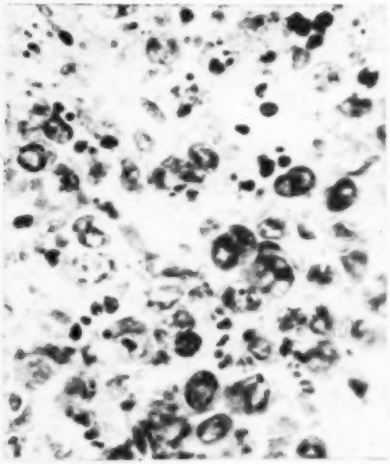

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Development of the tumour is favoured by oophorectomy, and inhibited by administration of oestradiol. Sometimes the tumour can be carried in series by intraperitoneal, subcutaneous or intravenous injection of ascitic fluid.

The author wishes to thank Professor C. E. Quensel, Statistical Institute, Lund, for help with the statistical analysis.

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CHANGES IN THE OVARIES OF MICE TREATED WITH DIMETHYLBENZANTHRACENE AND OBSERVATIONS ON THE SUBSEQUENT DEVELOPMENT OF TUMOURS IN OVARIES AND BREASTS

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Received for publication September 3, 1959

IT has previously been shown that fortnightly skin paintings of an oily solution of dimethylbenzanthracene (DMB) induce a high incidence of granulosa-celled tumours of the ovary, as well as of breast tumours, in mice of the IF strain and its first generation hybrids (Howell, Marchant and Orr, 1954 ; Marchant, 1957). The earliest ovarian tumour was encountered after 4 months of treatment.

In a subsequent experiment ovaries from F_1 C57 Bl × IF mice, which had been treated with DMB for 3 months, were transplanted bilaterally to the ovarian capsules of untreated mice. In 14 out of 18 of these animals large ovarian tumours were found 15 months later, many of them being luteinised (Marchant, 1959*a*). At the same time a preliminary account was given of the incidence of ovarian tumour nodules found in a series of DMB-treated mice killed at monthly intervals after beginning the treatment, for it was important to know whether or not incipient tumours were already present in the transplanted ovaries.

The present report gives further details of this attempt to discover at what stage in DMB treatment tumour nodules first appeared in the ovaries. It describes the histological changes that preceded the appearance of granulosa-celled tumours, as well as those that occurred simultaneously in non-tumourous ovaries. It also gives observations on the luteinisation of established granulosa-celled tumours, which was not seen in mice of the IF strain or C57 Bl strain reported previously (Marchant, 1957). Finally, it reports the incidence and histology of the palpable breast tumours which appeared after the 4th month of the experiment.

MATERIALS AND METHOD

Eighty-five virgin female mice lacking the mammary tumour agent were used in this experiment. They were F_1 hybrids derived from C57 Bl mothers and IF fathers. They were housed 5 in a box and fed on rat cubes known as the Thompson diet.

When the mice were between $2\frac{1}{2}$ and 4 months old, fortnightly skin paintings of 0·5 per cent DMB in olive oil were commenced. Up to 6 treatments were given, each mouse receiving about 0·2 ml. (1 mg.) distributed in 16 spots over dorsal and ventral surfaces at each painting.

Ten mice were killed at monthly intervals at the beginning of the experiment. After the 4th month breast tumours made their appearance, growing rapidly and necessitating the killing of some animals at irregular times. On account of this,

the results reported below include some mice which had to be killed within 2 weeks of the specified month.

For a short while before death, vaginal smears were taken from nearly all the mice surviving 4 months or more, to determine whether the ovaries were secreting oestrogen or not.

At autopsy the mice were examined for palpable breast tumours, many of which were removed for histological examination. All ovaries were examined and removed. Those from mice up to and including the 5th month of treatment were serially sectioned. After that time representative sections from several different levels were mounted. Sections were stained with haematoxylin and eosin.

The ovaries of 5 untreated mice aged 6 months were also examined and serially sectioned.

In the early stages follicles and corpora lutea in the largest sections from each ovary were counted under low power to give a rough quantitative estimate of changes.

RESULTS

Ovaries

Untreated mice.—The ovaries of the 5 untreated mice, which were 6 months old, were about 3 × 4 mm. in diameter, roughly kidney-shaped, coloured pale pink and studded with bright pink, white or pale yellow spots.

In histological sections, numerous follicles and corpora lutea were seen, bounded by a stroma of fibrous and vascular tissue (Fig. 1). The follicles occasionally contained 2 oocytes. They, together with the corpora lutea, were situated towards the periphery. A few tiny clusters of lipochrome cells were seen. The largest sections from each of the 10 ovaries were examined in detail. They contained from 12 to 22 follicles with granulosa-cells (mean 15·8) and 13–27 fragments of corpora lutea (mean 19·4) per section.

One month DMB treatment.—After 1 month of DMB treatment (2 fortnightly paintings) the ovaries of the 10 mice examined appeared similar to those of untreated animals.

Histologically, too, there was little difference from normal. The largest sections, however, contained slightly fewer follicles and corpora lutea. The follicles numbered 5–19 (mean 12·7) and the corpora lutea 9–24 (mean 17·5). In 4 of the animals a few of the corpora lutea appeared to be merging.

Two months DMB treatment.—After 2 months of DMB treatment the ovaries of the 10 mice killed appeared macroscopically normal. In histological sections, a slight increase in numbers of merging corpora lutea was seen and the numbers of follicles counted in the largest sections of each ovary had fallen considerably. The follicles numbered 0–8 (mean 3) and the numbers of individual corpora lutea were 8–23 (mean 15·5).

Three months DMB treatment.—After 3 months of DMB treatment (6 fortnightly paintings) only 1 mouse of the 10 killed had what appeared to be normal ovaries macroscopically. The other ovaries in the group were atrophied, fairly smooth and yellowish.

All 20 ovaries were very abnormal histologically (Fig. 2). In the largest sections no follicles were found, but examination of all the serial sections revealed

1 graafian follicle in 1 ovary of 4 different mice, and a 5th mouse had 3 graafian follicles in 1 ovary. Fusion of corpora lutea was much more marked in this group of animals and in 7 ovaries no distinct corpora were found. The ovaries of the mouse, which had appeared to be normal macroscopically, showed the least degree of fusion of corpora. In 1 mouse, in which no follicles were found and fusion of corpora lutea was well advanced, a nodule of tissue resembling granulosa-celled tumour was found, similar in size to a ripe follicle or corpus luteum. It is considered that this was the earliest certain tumour nodule found in this experiment. Four ovaries with fused corpora lutea contained tiny cysts.

Four months from 1st DMB treatment.—After 4 months of DMB treatment (6 fortnightly paintings) all the 10 mice killed had abnormal ovaries. In 3 mice the 2 ovaries were unequal in size, 1 ovary being about normal in size, the other slightly atrophied, yellowish and smooth. In the larger ovaries of these 3 mice small granulosa-celled tumours were found, the largest being nearly 2 mm. diameter and making up about half the volume of the ovary. These small tumours were relatively undifferentiated or showed pseudofollicular differentiation. One was made up of 2 small nodules, 1 of which is illustrated in Fig. 4 and 5. Vaginal smears of all 3 mice showed predominantly oestrus smears about the time of death.

In the other 7 mice, both ovaries were slightly atrophied, yellowish and smooth. Histologically these ovaries showed a complete absence of follicles and oocytes from all sections. Corpora lutea were rarely distinct bodies and most of them were fused into masses of lutein tissue. From this time onwards considerable variation began to occur in the types of cells comprising the lutein tissue. Sometimes the cells were vacuolated or pigmented and in some cases the lutein tissue was converted in part to collagenous tissue, resembling human corpora albicantia. This condition has been referred to as " hyalinised " (Fekete, 1946; Atkinson, Dickie and Fekete, 1954). Vaginal smears showed predominantly dioestrus smears in 3 mice and anoestrus smears in the others. In one of the former, a tiny nodule suspected of being an incipient tumour was found.

Five months from 1st DMB treatment.—Fourteen mice were killed about 5 months from the 1st DMB treatment, 10 of which had palpable breast tumours. Macroscopically their ovaries were more variable than at 4 months. One had a dark red lump about 13 mm. in diameter in 1 ovary, which proved to be a blood clot on histological examination.

Another mouse had an enlarged ovary, about 5 mm. diameter, divided into 2 separate lobes. One lobe was pink and this proved to be a granulosa-celled tumour which was slightly luteinised. The other lobe, like the contralateral ovary, was very pale yellow and knobbly. This mouse had predominantly dioestrus vaginal smears.

Four mice had ovaries unequal in size, the larger in each case proving to contain a small granulosa-celled tumour. Three of these had oestrus smears and 1 had anoestrus smears.

One mouse had smooth, pale pink ovaries, about normal in size, which were found to be invaded by lymphocytes. This mouse had a thymic tumour and generalised lymphomatosis, a condition found in about 20 per cent of the mice killed from 5 months onwards.

The other 8 mice in this group had atrophied ovaries which were pale yellowish colour and smooth or knobbly. Like non-tumourous parts of tumourous ovaries,

on histological examination they showed diffuse luteinisation (Fig. 3) with varying degrees of hyalinisation, the knobbly ovaries being the most hyalinised. There was a complete lack of follicles, occasional small clumps of pigmented cells or scattered mast cells. Two of these mice had ovaries containing small granulosa-celled tumours and they had predominantly oestrus smears. Two others had suspected tumour nodules in them, 1 having dioestrus and the other anoestrus smears (Fig. 7 and 8). The other 4 mice with atrophied ovaries had anoestrus smears.

Six months from 1st DMB treatment.—Eleven mice were killed about 6 months from the 1st DMB treatment, 9 of them having palpable breast tumours.

One of these mice had a cyst 5 mm. in diameter in 1 ovary. It was filled with clear fluid and in its walls the ovarian tissue was embedded. This was found to be a luteinised tumour. The mouse had anoestrus smears.

Another mouse had ovaries unequal in size, the larger containing a small granulosa-celled tumour, the vaginal smears being oestrus.

A third mouse had ovaries about normal in size, pale yellow and studded with white spots. This appearance was due to very marked hyalinisation of corpora lutea. A small luteinising tumour was found in 1 ovary and the vaginal smears were predominantly oestrus.

The other 8 mice had smooth, yellowish atrophied ovaries. A small granulosa-celled tumour was found in 1 of them and another had a suspected tumour and oestrus smears. The remainder had diffusely luteinised ovaries with, on the whole, a greater degree of hyalinisation then after 5 months and an increased number of mast cells present. They had anoestrus smears, with the exception of 1 mouse which had predominantly oestrus smears and whose ovaries were almost completely hyalinised.

Seven months from 1st DMB treatment.—Fourteen mice were killed about 7 months after the 1st DMB treatment, 13 of these having palpable breast tumours. One mouse had a purple lump about 9 mm. diameter in 1 ovary, which proved to be a blood clot. The other ovary was atrophied and was found to be completely invaded by lymphocytes.

Another mouse had in 1 ovary a cyst about 8 mm. diameter filled with clear fluid and a luteinised tumour was embedded in its walls. It had anoestrus vaginal smears.

Five mice had 1 enlarged ovary measuring between 4 and 8 mm. diameter. All of these contained granulosa-celled tumours and in 2 there were signs of luteinisation of the tumours. Four of the 5 mice had predominantly oestrus smears and the other had anoestrus smears.

Five mice had unequal but un-enlarged ovaries, the smaller being atrophied. The larger ovary of 1 of these proved to be a blood-filled cyst, but the contralateral atrophied ovary contained a small granulosa-celled tumour. This mouse had oestrus smears. Another had both ovaries very heavily invaded by lymphocytes and its vaginal smears were oestrus. The bigger ovary may well have contained a tumour but it was impossible to be certain because of the lymphocytic infiltration. It is therefore scored as a suspected tumour. In the other 3 mice with unequal ovaries a small granulosa-celled tumour, was found in the larger ovary. In 1 case it was partly luteinised (Fig. 9 and 10). One of these mice also had a small tumour in the smaller ovary. This mouse had anoestrus smears and the other 2 had oestrus smears.

The remaining 2 mice had atrophied ovaries and 1 contained a small cyst lined by ciliated epithelium. Both had anoestrus smears. All atrophied ovaries were diffusely luteinised and hyalinised to varying degrees (Fig. 4). Mast cells were frequently present, as were patches of large pigmented cells.

Eight months from 1st DMB treatment.—Six mice survived 8 months from the first DMB treatment, all developing breast tumours.

A solid yellow tumour about 9 mm. diameter was found in 1 mouse. It was a pseudofollicular granulosa-celled tumour, showing all stages of luteinisation (Fig. 11 and 12). Vaginal smears were oestrus.

Another mouse had unequal ovaries, the larger being divided into a yellow and a dark red lobe, each about 3 mm. diameter. The dark red lobe, contained a slightly luteinised granulosa-celled tumour, and blood-filled cyst. This mouse had anoestrus smears.

A 3rd mouse had both ovaries slightly enlarged—about 4 to 5 mm. diameter. One was red and yellow and enclosed in a small cyst filled with clear fluid and the red part proved to be a blood-filled cyst. The other was yellow in colour and

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EXPLANATION OF PLATES

All figures show sections of ovaries of F_1 C57 Bl × IF mice treated fortnightly with dimethylbenzanthracene (DMB). Haematoxylin and eosin stained.

Fig. 1 to 4 show non-tumourous ovaries.

FIG. 1.—Ovary of untreated mouse aged 6 months. Follicles in various stages of development and corpora lutea are present. × 28.

FIG. 2.—Ovary of mouse aged 5½ months which had received 3 months DMB treatment. All follicles have gone, but degenerate remains of oocytes are still present. × 32.

FIG. 3.—Ovary of mouse aged 7 months which had begun DMB treatment 5 months previously. Few degenerate oocytes remain, corpora lutea are fused and traces of hyalinisation are present. × 28.

FIG. 4.—Ovary of mouse which had begun DMB treatment 7 months earlier. There is a marked degree of hyalinisation of lutein tissue. × 28.

Fig. 5 to 8 show lesions suspected of being early granulosa-celled tumours.

FIG. 5.—Ovary of mouse which had begun DMB treatment 4 months earlier. Amongst eosinophilic lutein tissue, a small nodule of more basophilic cells is seen. A larger separate nodule was found in this ovary. Vaginal smears were oestrus. × 60.

FIG. 6.—Same nodule as Fig. 5 under greater magnification. Nuclei of many of the cells show prominent nucleoli and nuclear membrane, typical of larger tumours (see Fig. 12). Granulosa cells of normal follicles have nuclei which stain intensely with haematoxylin and in which no structure can be seen. × 120.

FIG. 7.—Part of a diffusely luteinised ovary containing the smallest suspected tumour nodule found. Five months after DMB treatment began. Vaginal smears anoestrus. × 60.

FIG. 8.—Same nodule as Fig. 8. The cells show a whorled arrangement with fibroblasts separating them and more basophilic cytoplasm than surrounding lutein tissue. This whorled arrangment is typical of some of the undoubted early tumours seen in these hybrid mice. × 120.

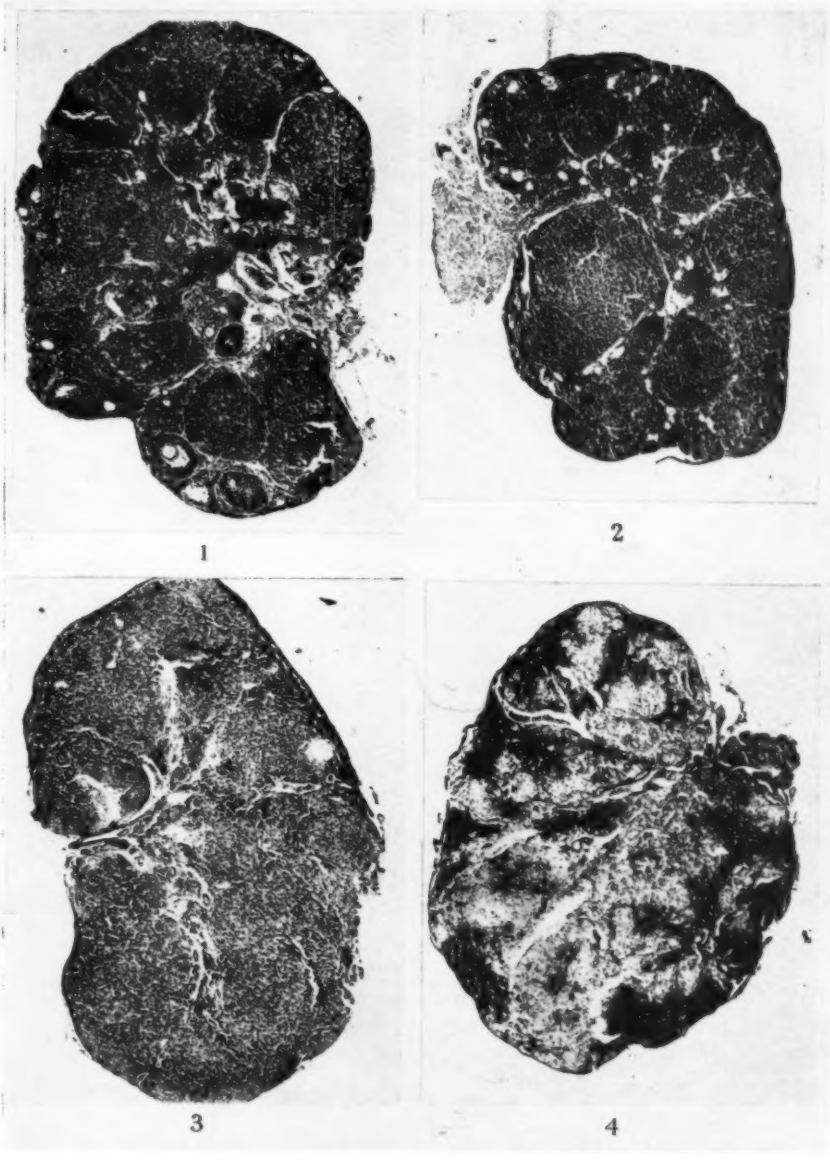
Fig. 9 to 12 show ovaries entirely made up of tumour and undergoing luteinisation.

FIG. 9.—Small ovarian tumour from a mouse killed 7 months after first DMB treatment. A portion of the tumour is still granulosa-celled, but the greater part of it is considerably luteinised. Vaginal smears oestrus. × 28.

FIG. 10.—A luteinised area from the same tumour as Fig. 9. Some of the luteinised cells are pigmented. × 120.

FIG. 11.—Part of a large ovarian tumour found 8 months after DMB treatment began. Cells of undifferentiated granulosa-celled tumour in nodules are surrounded by areas of luteinised tumour cells. Vaginal smears oestrus. × 28.

FIG. 12.—Part of Fig. 11 at higher magnification. Cells typical of undifferentiated granulosa-celled tumour can be seen adjacent to heavily luteinised tumour cells. × 120.



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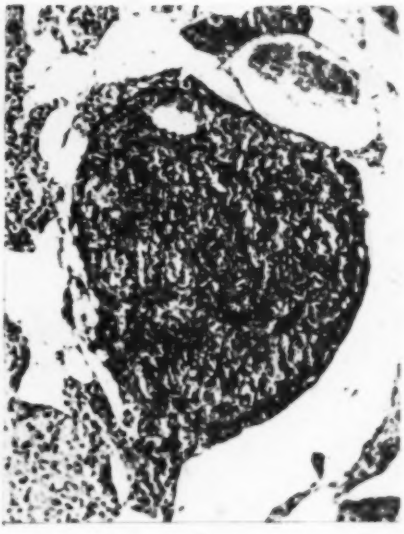

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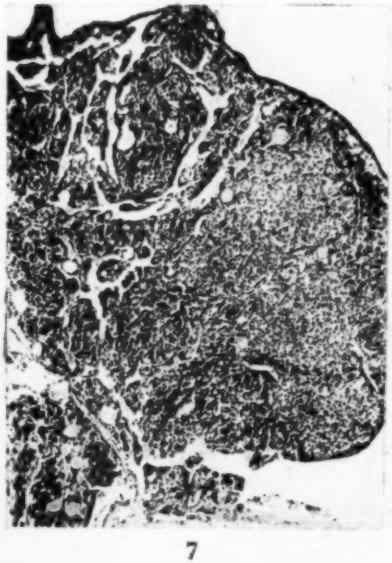

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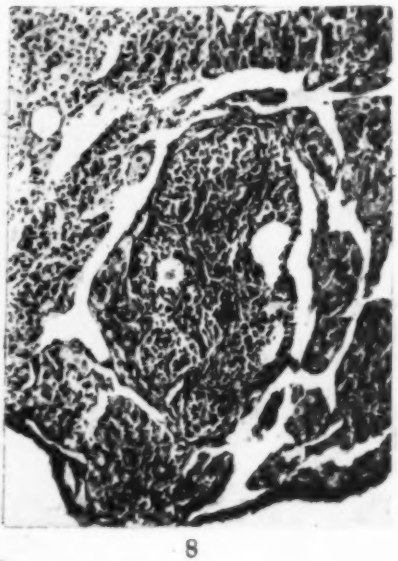

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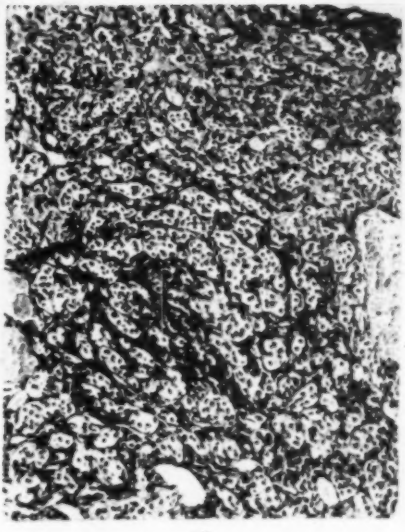

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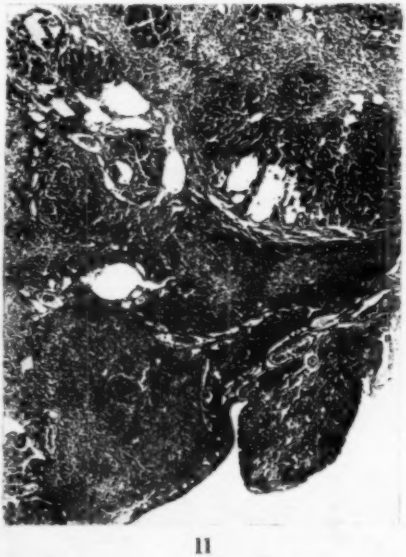

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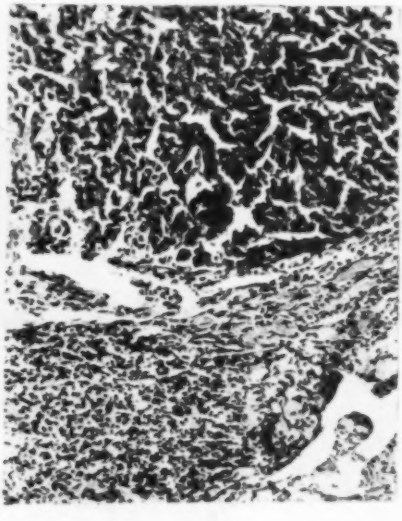

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diffusely luteinised, with a small pseudofollicular granulosa-celled tumour in it. Vaginal smears were predominantly oestrus.

The other 3 mice had atrophied ovaries. These were all diffusely luteinised, with pigmented cells and prominent germinal epithelium. In 1 case, the latter was invaginated, and there was slight invasion of the ovaries by lymphocytes. In another very small ovary, there were clusters of anovular follicules and an invaginating germinal epithelium. Both of these mice had predominantly dioestrus smears. The 3rd had a small granulosa-celled tumour in 1 ovary and a suspected tumour nodule in the other. Its smears were anoestrus.

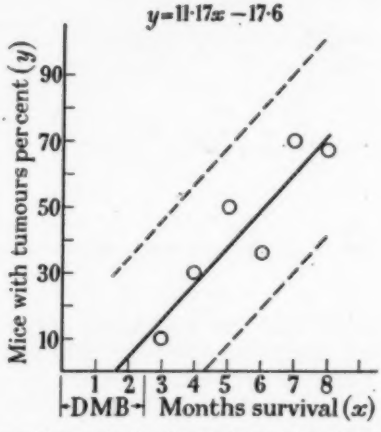

FIG. 13.—Increase of detectable neoplastic changes in ovaries of DMB-treated mice with time.

Ovarian tumour incidence and survival.—The incidences of granulosa-celled tumours which occurred in the different groups of mice is given in Table I. The incidence has been plotted against survival time in Fig. 13 and it can be seen that

TABLE I.—*Incidence of Granulosa-celled Ovarian Tumours by Size per Month since 1st DMB Treatment*

Months since 1st DMB	Total mice	Without tumours	Suspected tumour nodules	Histologically detected tumours	Tumours in unequal ovaries	Tumours in enlarged ovaries	Mice with ovarian tumours (per cent)
0	5	5	0	0	0	0	0 (0)
1	10	10	0	0	0	0	0 (0)
2	10	10	0	0	0	0	0 (0)
3	10	9	0	1	0	0	1 (10)
4	10	6	1	0	3	0	3 (30)
5	14	5	2	2	4	1	7 (50)
6	11	6	1	2	2	0	4 (36)
7	14	3	1	2*	4*	5	10 (70)
8	6	2	1*	1*	0	3	4 (67)
Total	90						

* Bilateral tumours.
† Excluding suspected tumour nodules.

there is a roughly linear increase of incidence with time. The regression line and its 95 per cent confidence limits are shown on the figure.

Table II summarises month by month the main histological structures found in the ovaries of the C57 Bl × IF hybrid mice treated with DMB.

TABLE II.—*Structures Present in Ovaries of F_1 Hybrid C_{57} Bl × IF Mice Treated with DMB*

Months DMB	Follicles	Corpora lutea			Ovarian tumours		
		Separate	Fused	Hyaline	Suspected	Granulosa-celled	Partly luteinised
0	++	++	−	−	−	−	−
1	++	++	±	−	−	−	−
2	+	+	+	−	±	−	−
3	±	+	+	−	−	±	−
4	−	±	++	+	±	+	−
5	−	−	++	+	±	++	−
6	−	−	++	+	±	+	±
7	−	−	+	+	±	+	+
8	−	−	+	−	±	+	+

++ Abundant. + Present. ± Sometimes present, sometimes absent. − Absent.

Breast tumours

Breast tumours occurred after the 4th month from the beginning of DMB treatment. After the 5th month, many of the mice had more than 1 palpable tumour when killed. Table III gives the numbers of tumours developed per mouse and the incidence of mice with palpable breast tumours.

TABLE III.—*Incidence of Mice with Palpable Breast Tumours per Month Since First DMB Treatment*

Months DMB	Total mice	Number of palpable breast tumours per mouse					Mice with breast tumours (per cent)
		1	2	3	4	5	
0–4	50	0	0	0	0	0	0 (0)
5	14	9	1	0	0	0	10 (71)
6	11	4	3	2	0	0	9 (82)
7	14	3	6	0	2	2	13 (93)
8	6	1	1	4	0	0	6 (100)

Over 40 breast tumours were examined histologically. They were adenocarcinomas, often of a papillary type, and frequently with a marked fibroblastic component. A little secretion in the tubules was generally present. Squamous metaplasia was seen in a few tumours, but it was very small in amount. Slight sebaceous metaplasia was sometimes seen.

Table IV, V and VI show the relationship between the presence of ovarian secretion (as judged by vaginal smears about the time of death), ovarian tumours (including suspected tumours) and palpable breast tumours. χ^2 tests on the data of these tables show a significant correlation between the presence of ovarian tumours and the secretion of ovarian hormones, but not between ovarian tumours and breast tumours, or between breast tumours and ovarian secretion. Only

TABLE IV.—*Relative Incidence of Breast and Ovarian Tumours in 45 C57 Bl × IF mice Treated with DMB*

		Breast tumours		
		+	−	Totals
Ovarian tumours	+	27	3	30
	−	11	4	15
Totals		38	7	45

$\chi^2 = 2{\cdot}593$. $P > 0{\cdot}1$.

TABLE V.—*Relation between Presence of Ovarian Tumours and Ovarian Secretion as Judged by Vaginal Smears in 42 C57 Bl × IF Mice Treated with DMB*

		Vaginal smears		
		Oestrus or dioestrus	Anoestrus	Totals
Ovarian tumours	+	21	8	29
	−	3	10	13
Totals		24	18	42

$\chi^2 = 8{\cdot}9$. P about $0{\cdot}003$.

TABLE VI.—*Relation between Presence of Ovarian Secretion and Breast Tumours in 42 C57 Bl × IF Mice Treated with DMB*

		Vaginal smears		
		Oestrus or dioestrus	Anoestrus	Totals
Breast tumours	+	22	13	35
	−	2	5	7
Totals		24	18	42

$\chi^2 = 2{\cdot}224$. P about $0{\cdot}12$.

mice which survived over 4 months are included, because breast tumours were not apparent before this time.

DISCUSSION

Ovarian grafting-experiment

It will be seen from Table I that, in the 10 mice killed 3 months after the first DMB painting, only 1 early tumour was found in serial sections of all 20 ovaries. We may be fairly sure, then, that detectable incipient ovarian tumours were present in very few of such ovaries transplanted to the 18 mice in the original experiment (Marchant, 1959*a*). However, the fact that 14 of them (78 per cent) developed large ovarian tumours indicates that changes leading towards tumour production had already occurred in the majority of ovaries after 3 months treatment. The present histological study showed no evidence of any form of hyperplasia, but rather of ovarian atrophy brought about by follicular destruction with accompanying reduction in numbers of corpora lutea. In a more recent experiment the mean weight of ovaries from normal young adult F_1 C57Bl × IF mice was

found to be 9·8 mg., while that of ovaries removed 3 months after commencing DMB treatment was only 4 mg.

The fact that few really large ovarian tumours were found in the present experiment is undoubtedly due to the time factor involved. Breast tumours developed in rapidly increasing numbers after 4½ months of treatment and this made it necessary to kill all animals by the 8th month. In the original experiment (Marchant, 1959*a*) the ovaries grafted from DMB treated to normal animals after 3 months of treatment were able to survive a further 14 months in their new hosts, allowing ample time to grow into really large tumours.

Histogenesis of ovarian tumours

When we consider the histogenesis of the granulosa-celled tumours, it seems impossible to say what type of tissue the tumours originated from. Fig. 13 shows that ovarian tumours steadily continued to appear after 3 months DMB treatment, although by 4 months no follicles remained in any ovaries. It would seem that tumours which arose after 4 months treatment could not have arisen from follicular tissue.

There was no evidence of origin of the granulosa-celled tumours from down-growth and invagination of germinal epithelium, such as precedes the appearance of tubular adenomas, described by Russell and Fekete (1958). The prominence of germinal epithelium and slight invagination of it was only occasionally seen in the late stages of the experiment and seemed to be associated with ovarian atrophy.

It is possible that the tumours may have arisen from stem cells in the ovarian parenchyma, a view held by Willis (1953) and others.

Luteinisation of granulosa-celled tumours

From a study of the tumours in this and other experiments (Marchant, 1959*b*) with ovaries from DMB-treated mice, it seems quite clear that luteomas arise from pre-existing granulosa-celled tumours. At the time when granulosa-celled tumours are first detected, the ovaries are usually in a diffusely luteinised condition resulting from fusion of corpora lutea, with fusion and vacuolation of their cells. This diffusely luteinised tissue somewhat resembles that of a granulosa-celled tumour that has undergone a marked degree of luteinisation. Thus, on seeing a diffusely luteinised ovary containing an incipient granulosa-celled tumour, one might be tempted to assume that granulosa-celled tumours arise in pre-existing luteomas. That this is not the case can only be determined by study of a series of animals killed at intervals of time throughout the induction period. The series described here has shown that ovaries in a diffusely-luteinised condition are atrophied and do not grow in size, in fact mitosis has never been observed in a luteinised cell.

The luteinisation of granulosa-celled tumours was not seen in IF or C57 Bl mice (Marchant, 1957), but it was very marked in the grafting experiment already mentioned (Marchant, 1959*a*) utilising F_1 C57 Bl × IF mice. It was also seen in the later stages of an experiment in which IF or C57 Bl ovaries were transplanted into C57 Bl × IF hosts prior to DMB-treatment (Marchant, 1959*b*). It may be that luteinisation is a matter of maturation of the tumour cells, or it may be brought about by some factor in the internal environment of the hybrid mice differing from that in the parent strains.

Hyalinisation

"Hyalinisation" of corpora lutea was a phenomenon not previously encountered in ovaries of C57 Bl mice treated with DMB and only rarely in mice with IF ovaries (Howell, Marchant and Orr, 1954). It was frequently seen in these C57 Bl × IF hybrid ovaries as in old dba mice (Fekete, 1946) and in dba × CE hybrid mice (Atkinson *et al.*, 1954).

Hormone production by ovarian tumours

Secretion of oestrogen, as judged by vaginal smears, was seen in 21/29 (72 per cent) of animals with ovarian tumours (Table V). This is comparable with the 39/48 (81 per cent) found in the previous report of ovarian tumours in IF or IF hybrid mice (Howell, Marchant and Orr, 1954) and indicates that approximately 1 out of 4 or 5 granulosa-celled tumours in mice does not secrete oestrogen.

Breast tumours

The DMB treatment induced breast tumours in the majority of mice surviving more than 4 months. There was no correlation between the presence of breast tumours and oestrogen secretion at the time of death, as shown by Table VI.

SUMMARY

Eighty-five virgin female F_1 hybrid mice between C57 Bl and IF strains were used. They were given up to 6 fortnightly skin paintings of 1 mg. DMB in olive oil in order to induce ovarian tumours. As far as possible, 10 mice were killed at monthly intervals and their ovaries sectioned and examined histologically.

The first change noticed in all ovaries was a gradual diminution in numbers of follicles until all had disappeared by the 4th month. Corpora lutea became fused, until by the 4th month there were rarely any distinct ones to be found. After this, lutein tissue showed considerable variation with frequent vacuolation and pigmentation of cells. Sometimes it became converted to collagenous tissue resembling human corpora albicantia. As a result of these changes the ovaries atrophied in size.

Incipient granulosa-celled tumours were found from the 3rd month onwards. They were usually unilateral and were detected in a steadily increasing proportion of animals. About three-quarters of the tumours secreted oestrogen, as judged by vaginal smears. In the later stages the tumour cells showed traces of luteinisation.

There was a gradual increase in numbers of palpable breast tumours after the 4th month.

The author is grateful to the Birmingham Branch of the British Empire Cancer Campaign for support of this work.

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THE INFLUENCE OF PSEUDOPREGNANCY ON THE INDUCTION OF MAMMARY TUMOURS BY METHYLCHOLANTHRENE IN MICE OF THE BALB/c STRAIN

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Received for publication September 7, 1959

MAMMARY carcinomas can be induced in virgin IF female mice by means of chemical carcinogens (Bonser, 1958). But it was shown by Bonser (1954) that oestrogen alone was not a sufficient substitute for the ovaries as the hormonal stimulus in the induction of such tumours by methylcholanthrene. However, by replacement of the ovarian steroid hormones by oestrogen and progesterone, Jull (1954) was able to obtain 9 tumours in 11 ovariectomised mice. He also demonstrated (1953) that virgin females of this strain have a well-developed duct and acinar system, the latter reaching a maximum at about 18 weeks of age but showing much individual variation. This extensive acinar development and the demonstration by van der Lee and Boot (1955) that spontaneous pseudopregnancy is frequent in virgins of some mouse strains when the females are caged in groups, suggested that high levels of progesterone might be operating in the IF female and might account for the high incidence of breast tumours induced by chemical carcinogens. It was therefore decided to test the effect of repeated induced pseudopregnancies on tumour induction by a chemical carcinogen.

The strain chosen was the BALB/c, which does not carry the milk factor (Andervont, 1940) and has a low incidence of spontaneous mammary cancer. The only information available regarding the reaction of this strain to chemical carcinogens was that obtained by Squartini (1958) who had failed to induce mammary tumours in 30 virgin mice by means of subcutaneous injection of 1 mg. of 20-methylcholanthrene in 0·2 c.c. of olive oil. There was, therefore, no guide in regard to the dose and duration of the chemical treatment to be adopted. In order to gain an insight into the mode of action of the chemical, it was decided to test three groups of mice : virgins kept five in a cage ; virgins deprived of the olfactory lobes and kept singly in order to reduce pseudopregnancy to a minimum (van der Lee and Boot, 1956) ; and virgins kept with vasectomised males in order to induce pseudopregnancy.

MATERIALS AND METHODS

BALB/c strain

A litter of this strain was given to the Division of Cancer Research, University of Perugia, by the Chester Beatty Research Institute, London, in November, 1953. It was then in the 79th generation of inbreeding. The Chester Beatty

Institute had previously obtained the strain from L. Dmochowski, Department of Cancer Research, Leeds, in December, 1952, when it had reached 76 inbred generations. The donor to Leeds was H. B. Andervont.

During the period in Perugia, this strain has reached the 99th generation of inbreeding and has shown a low mammary cancer incidence. During 1958, in 10 virgins there were no tumours and in approximately 40 breeders there were 7·5 per cent, the latent period being 76 weeks or more. During 1959, no tumours occurred in 44 virgins, but in 44 breeders the incidence was 7·0 per cent at the same late date. In the hands of Andervont (1941) this strain had an incidence of mammary cancer in breeding females of less than 2 per cent.

Group I (36 mice).—At 4–5 weeks of age, virgin mice were placed five in a cage and were so kept throughout the experiment.

Group II (41 mice).—At approximately 6 weeks of age, under ether anaesthesia, the olfactory lobes were removed surgically from virgins by means of suction through small trephine holes in the anterior part of the cranium immediately on either side of the mid-line. The mortality was low (10 per cent). After recovery the mice were kept five in a cage for approximately one week, but thereafter they were placed singly in cages.

Group III (32 mice).—At 6 weeks of age, groups of three virgins were mated with one vasectomised male, which was allowed to remain in the cage throughout the experiment.

All groups received similar chemical treatment, namely six applications to the skin at fortnightly intervals of 16 drops of 0·5 per cent 20-methylcholanthrene (supplied by Messrs. Light & Co. Ltd., Colnbrook, Bucks) in almond oil (8 drops on the dorsal and 8 on the ventral surface) commencing at 12 weeks of age. It was computed that 1 ml. of oil, containing 5 mg. of carcinogen, was used for each application and that the mice were thus exposed to the carcinogen for a period of 12 weeks. The animals stood the treatment well.

A diet of cubes (supplied by Messrs. Pilsbury, Birmingham) and water *ad lib.* was given.

At post mortem a whole mount was prepared of the third left (thoracic) breast of each treated mouse. The ovaries with capsule were weighed wet and the uterine horns were assessed by naked eye examination as normal, increased or decreased in size.

RESULTS

Mammary tumours

(a) *Incidence.*—The date of appearance of the mammary tumours and the survival of the non-tumourous mice are shown in Fig. 1. All the tumours were single, except in two mice of Group III. In one of these, three tumours developed at 25 weeks and in the other two tumours at 37 weeks. In Table I the percentage of mice bearing mammary tumours in relation to survival after the initiation of treatment is given. No tumours occurred in virgins (Group I), 2·4 per cent in lobectomised virgins (Group II) and 43·8 per cent in females mated with vasectomised males (Group III). The survival rate was considerably shorter in the last group, 4 of 32 mice surviving for more than 32 weeks, whereas the survival was 35 out of 36 and 33 out of 41 in the other two groups respectively (Fig. 1).

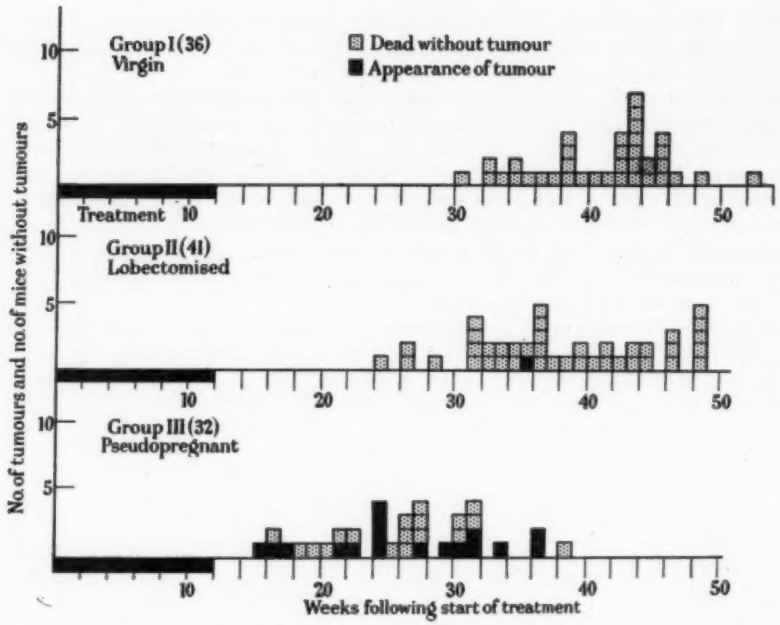


FIG. 1.—Incidence of mammary tumours.

EXPLANATION OF PLATES.

FIG. 2.—Irregular tubular mammary carcinoma (Group III) appearing 25 weeks after the beginning of treatment. The tubules are irregular in size and shape and there is little stroma except at bottom right. × 60.

FIG. 3.—Solid polygonal cell mammary carcinoma (Group III) appearing 22 weeks after the beginning of treatment, with advanced squamous metaplasia. × 60.

FIG. 4.—Papillary cystic mammary carcinoma (Group III) appearing 32 weeks after the beginning of treatment. × 60.

FIG. 5.—Whole mount of third left breast (Group I) 31 weeks after the beginning of treatment. A well-developed pattern of dilated ducts is seen, with no lobules but numerous end buds. × 60.

FIG. 6.—Whole mount of third left breast (Group III) 24 weeks after the beginning of treatment. Florid acinar development. This mouse had a mammary tumour in another breast. × 60.

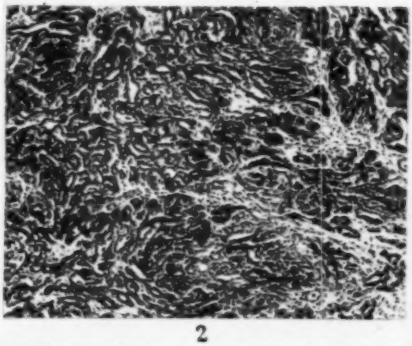
FIG. 7.—Whole mount of third left breast (Group III) 21 weeks after the beginning of treatment. Nodule at top. × 60.

FIG. 8.—Ovary (Group I) 41 weeks after the beginning of treatment. Atretic follicles round the edge. Remains of corpora lutea in the interstices with a group of dark-staining cells in the centre. Pigment-containing phagocytes below centre and top right. × 60.

FIG. 9.—Ovary (Group III) 27 weeks after the beginning of treatment. Atretic follicles top right and bottom left. Numerous large, old corpora lutea. × 60.

FIG. 10.—Longitudinal section of uterine horn (Group I) 45 weeks after the beginning of treatment. Lumen narrow with simple crypts dipping into stroma and simple tubular glands. × 60.

FIG. 11.—Longitudinal section of uterine horn (Group III) 27 weeks after the beginning of treatment. Lumen irregular with irregular crypts dipping into stroma. Large number of glandular cross sections. × 60.



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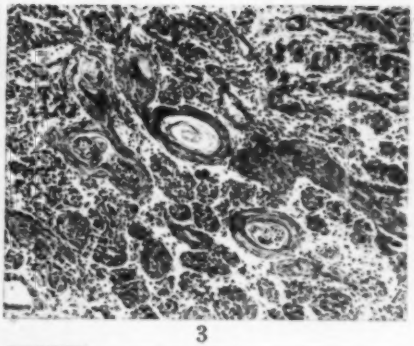

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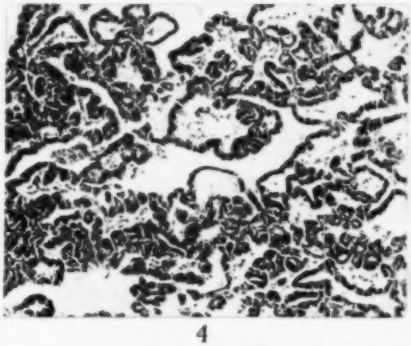

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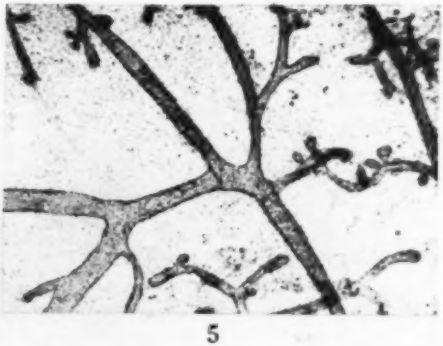

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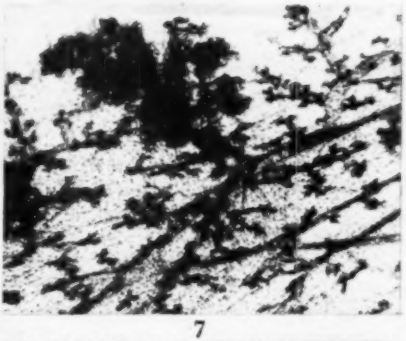

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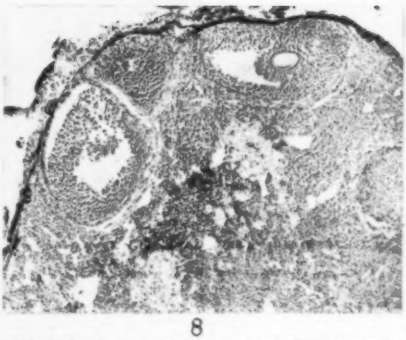

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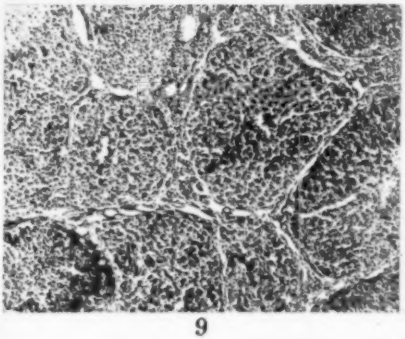

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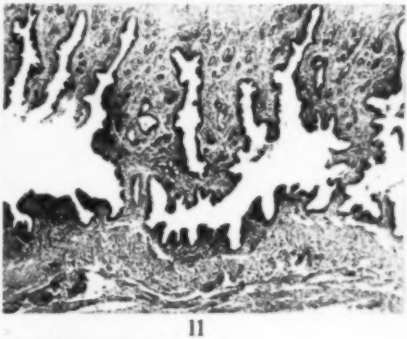

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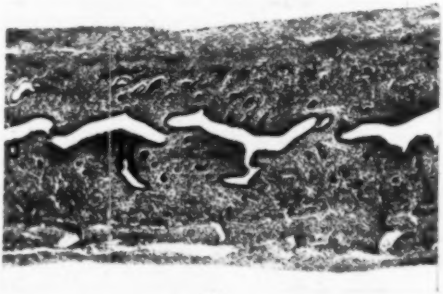

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TABLE I.—*Incidence of Mammary Tumours in Mice Surviving to the Beginning of Each Ten-week Period*

Group	Weeks of survival following initiation of treatment					Total	Tumours (per cent)
	10–19	20–29	30–39	40–49	50–59		
I	36	36	36	22	1	0/36	0
II	41	41	1/37	18	0	1/41	2·4
III	3/32	5/29	6/11	0	0	14/34	43·8

Numerator = number of mice bearing mammary tumours.
Denominator = number of mice surviving to the beginning of the stated period.

(b) *Histological structure.*—Using the classification adopted by Bonser (1954), the mammary tumours in Group III were classified as follows (Fig. 2–4) : irregular tubular 11 (6 with squamous metaplasia and five without), solid polygonal cell four (all with squamous metaplasia), and two papillary cystic tumours (one with early squamous metaplasia). No carcinosarcomas occurred. The one tumour which appeared in Group II was of solid polygonal-cell type, with squamous metaplasia. Emboli of tumour cells were present in some of the perivascular lymphatics of one lung.

Structure of the breast

This was assessed by the examination of the whole mounts. No differences could be detected in the breasts of Groups I and II. The duct system was well developed, individual ducts were generally slightly dilated, subsidiary ducts were small in number, end buds were prominent but acini were few (Fig. 5) and only in two mice were small lobules seen. In two other mice single " nodules " were seen. By contrast, the breasts of Group III showed florid acinar development, the main ducts were often greatly dilated (especially towards the nipple) and nodules were present in all but one of the mice (Fig. 6 and 7). The latter were numerous, except in 4 mice in which one or two nodules only occurred.

Skin Tumours

Squamous papillomas and carcinomas of the skin occurred very frequently in any site in all groups. The larger carcinomas usually ulcerated and thus reduced the life span of the animal.

Lung tumours

Pulmonary adenomas, usually multiple, occurred in all groups usually remaining small in size.

Ovaries

(a) *Weight.*—Those of Group III were significantly heavier than those of the other two groups (Table II). The range in all groups was considerable.

(b) *Histological structure.*—There was a marked difference in the appearance of the ovaries in Groups I and II compared with Group III. In the former, atretic follicles in various phases and broken-up remnants of corpora lutea were seen (Fig. 8). Entirely normal follicles were absent, though some young follicles

TABLE II.—*Ovarian Weights and Uterine Volume*

Group	Ovary			Uterus			
	Number of mice	Average (mg.)	Range (mg.)	Number of mice	Increased volume	Normal volume	Decreased volume
I	36	0·011	0·003–0·027	36	10	10	16
II	41	0·010	0·003–0·020	41	2	8	31
III	32	0·018	0·009–0·030	32	26	5	1

showed only a damaged ovum. Corpora lutea were present only in occasional mice of Group I. In the ovaries of Group III, atretic follicles were present in numbers approximating to those in the other two groups, as well as large numbers of large corpora lutea (Fig. 9). The lutein cells stained dark pink with eosin and were judged to be old structures. Although occasional luteomatous proliferations were seen in the ovaries of all groups, no ovarian tumours were detected.

Uterus

(a) *Volume.*—The uterus was inspected at post mortem and an assessment was made of the size of the horns (Table II). In Group I the horns were either thread-like, normal or slightly increased in volume ; in Group II they were usually thread-like, whereas in Group III they were often thick and dilated.

(b) *Histological structure.*—The uteri of Groups I and II had a structure characteristic of the virgin mouse, the endometrium being composed of a single lining layer of columnar cells placed on dense stroma, into which dipped small numbers of simple tubular glands. The lumen was narrow, with occasional crypt-like depressions into the stroma (Fig. 10). The uteri of Group III showed a very different picture. There was no excess of stroma and the component cells were not swollen, but the amount of surface epithelium was greatly increased by numerous irregular clefts which dipped into the stroma and gave the lumen a papillary aspect (Fig. 11). The number of cross-sections of tubular glands was increased, due either to an increase in the actual number of glands or to a more complicated structure of those present. Penetration of the endometrial glands into the muscular coat did not occur.

DISCUSSION

The present experiments have demonstrated that intact or lobectomised virgins of the BALB/c strain, which is free of the milk factor and has a low spontaneous incidence of mammary cancer, do not develop mammary tumours when treated with a dose of methylcholanthrene which is known to be in excess of that required to induce tumours in virgins of the IF strain. Jull (1956), using a standard limited dose of 4 applications to the skin of an oily solution of the compound, obtained 6 mammary tumours in 16 IF mice (38 per cent).

When pseudopregnancy was induced by mating females with vasectomised males (Group III) 44 per cent developed mammary tumours, the induction period ranging from 16–37 weeks following the initiation of treatment, a result closely comparable to that obtained by Jull in IF virgins. Thus the hormonal conditions of pseudopregnancy act as a promoting agent for the induction of mammary tumours in BALB/c breasts already submitted to the initiating action of a carcin-

ogen. This type of promoting stimulus is not essential for the induction of mammary tumours in this strain by the milk factor for Severi, Olivi and Biancifiori (1958) found an incidence of 54 per cent of mammary tumours in *virgins* at an average age of 50 weeks in BALB/c mice which had been given milk factor by Andervont in 1940 (called BALB/c+ in Perugia).

The florid structure of the breasts, the large number of corpora lutea in the ovaries and the hyperplastic state of the endometrium constitute evidence that the mice placed with vasectomised males were under the influence of oestrogen-progesterone secretion. Thus these experiments provide further evidence that the carcinogenic action of methylcholanthrene on the mouse breast is augmented by oestrogen combined with progesterone.

The absence of tumours in intact and lobectomised virgins was not due to short survival in these groups, for they lived a good deal longer than the pseudopregnant mice (Fig. 1). It might be suggested that pseudopregnancy alone was the cause of the mammary tumours and that initiation by the chemical was an unnecessary factor. This seems unlikely, as mammary tumours occur spontaneously in low yield only, in old breeders of this strain. It is a point which is, however, under investigation.

No attempt was made to study the histogenesis of the mammary tumours in Group III but the presence of large numbers of typical hyperplastic nodules in the breasts is in keeping with van Rijssel's (1956) demonstration that in mice with the milk agent there is a relation between the number of these structures and the subsequent appearance of the fully formed tumours. He calculated that about 60 nodules were necessary for every palpable tumour that presented.

The morphology of the induced tumours was similar to that of chemically-induced tumours of the IF strain. The predominant tumour was the irregular tubular adenocarcinoma, which may be regarded as the characteristic tumour of chemical induction, but solid polygonal and papillary cystic tumours also occurred. In two thirds there was squamous metaplasia, thought by Bonser (1958) to be associated with excessive dosage of the chemical. Although carcinosarcomas were not found, this type of tumour is not uncommon in IF mice treated with methylcholanthrene or 1 : 2 : 5 : 6-dibenzanthracene.

SUMMARY

Three groups of females of the BALB/c strain (with low breast cancer incidence and no milk factor) were treated with 20-methylcholanthrene applied to the skin in oily solution.

In Group I (36 virgins kept 5 in a cage) no mammary tumours occurred, although the mice survived for a period greater than 30 weeks after the beginning of treatment. In Group II (41 virgins with olfactory lobes removed) there was one mammary tumour 36 weeks following the initiation of treatment, 37 mice having survived for 30 weeks or more. In Group III (32 females kept 3 in a cage with a vasectomised male) the incidence of mammary tumours was 43·8 per cent, the latent period being 16–37 weeks. Benign and malignant tumours of the skin and lung adenomas occurred in all groups.

In the mice of Group III there was evidence of the excessive hormonal stimulation of pseudopregnancy in the florid structure of the breasts, the large number of corpora lutea in the ovaries and the hyperplasia of the uterine endometrium.

It is postulated that the hormonal stimulation of pseudopregnancy, through oestrogen and progesterone, acted as the promoting agent in the causation of mammary tumours in breasts which had been subjected to the initiating action of methylcholanthrene.

C. Biancifiori and F. Caschera were supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Maryland, U.S.A.

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TISSUE REGENERATION AND TUMOUR DEVELOPMENT

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Received for publication October 2, 1959

THE early changes, including hyperplasia, induced in the liver of the rat by the oral administration of 2-acetylaminofluorene were described by the author and his French colleagues (Laws, Mabille, Royer and Rudali, 1952) and in less detail by Skoryna and Webster (1951). The studies have been continued for some years in this department. Recently Laird and Barton (1959) have investigated the quantitative aspect of this effect and have pointed out that the period up to the onset of hyperplastic change in the liver of animals treated with this carcinogen appears to coincide with the minimum period of administration of the chemical which is necessary to provoke the ultimate appearance of tumours in this organ. The work described here has made use of the possibility of inducing regenerative hyperplasia in the liver by means of partial hepatectomy to study in more detail the influence of such a reaction on the effect of 2-acetylaminofluorene. Histological examination of the liver during the process has helped to make clear some of the mechanisms involved.

MATERIALS AND METHODS

Animals.—The rats used in most of this work were bred in this laboratory and were of Wistar stock. In the experiment contrasting the behaviour of young and old rats, the animals were of Birmingham stock, obtained from the Department of Anatomy, University of Birmingham. No difference has been noticed in the response of these two stocks of rats to treatment with 2-acetylaminofluorene. Only male rats were used throughout, since liver changes are rarely found in females. Unless noted all rats were adult (weight at least 200 g.) when the experiments began.

Carcinogen treatment.—The carcinogen, 2-acetylaminofluorene was obtained in pure form from L. Light and Sons. It was used, at a strength of 0·1 per cent incorporated in a meal diet supplied by the North Eastern Agricultural Co-operative Society, Aberdeen. The carcinogen was dissolved in 500 ml. of acetone and mixed with 10 kg. of the meal which became lightly dampened throughout. Water was added to form a paste which was formed into cakes and dried. These were fed *ad libitum* to the rats together with water. The meal used was of the same composition as the rat cakes from the same manufacturers used to feed the general stock of rats and mice, and provided a full balanced diet.

Partial hepatectomy.—The operation, by the method of Higgins and Anderson (1931), consisted of the removal of the anterior and left anterior lobes of the liver after ligature. Approximately two-thirds of the liver substance was effectively removed, on the average.

General.—All animals included in the experimental figures were subjected to a full post-mortem examination and histological sections of all relevant lesions were examined after staining with haematoxylin and eosin.

RESULTS

The earliest changes provoked by 2-acetylaminofluorene are of a toxic nature, reducing the efficiency of the liver cells and leading to the death of many cells. In earlier experiments of the author (Laws *et. al.*, 1952) and in those of Laird and Barton (1959) this was in some cases sufficient to lead to the death of the animals. Even in those rats less severely affected, histological examination of the liver showed a loss of parenchymatous cells around the portal tracts, with hyperplasia of small non-parenchymatous cells of uncertain origin. This stage is followed by one in which cell division among the parenchyma cells rapidly produces a replacement of the lost tissue, and goes on steadily to genuine hyperplasia. In the animals used in the present experiments (albino rats of so-called Wistar strain bred in the laboratory, but of uncertain ancestry), the changes are less dramatic, death seldom occurring during normal 2-acetylaminofluorene feeding. Histologically also the changes are more gradual but the outcome is the same, a nodular, hyperplastic liver. In spite of the little apparent change in the livers of these animals, partial hepatectomy performed after three weeks feeding unmasks a profound diminution in liver efficiency. If such animals are treated with the carcinogen after the operation also, most of them are dead within two weeks (26 out of 40 animals up to date). Such animals which die or are killed at this time are found to have liver weights only about one-third of the normal, i.e. only about the amount of liver left by the hepatectomy, in contrast with the rapid replacement of liver tissue in normal animals after such an operation. Ascites is invariably present and usually other effusions and a general waterlogging of the tissues. This probably results from hypoalbuminaemia due to poor liver function, although this has not so far been proved by direct estimation.

Histologically such livers present the series of changes noted above in a very severe form. Within a few days of the operation much of the parenchyma of the remaining lobes has disappeared, principally around the portal tracts, and there is marked proliferation of small cells in this region, stretching out into the parenchyma which is still present (Fig. 3). If the animal survives as long as a week, regeneration begins and can be seen by the eighth day by the naked eye in the form of small translucent nodules on the surface of the organ, and on the cut surfaces. Such nodules can be shown by a series of preparations examined histologically to originate as small groups of cells (presumably formed in the first place by division of one cell) staining basophilically and containing many mitotic figures (Fig. 1 and 2). They are always situated in the region of the radicles of the hepatic vein, the part least damaged, although they enlarge rapidly and by the fourteenth day may largely have replaced the remaining old parenchymatous cells, and the small cells which had infiltrated (Fig. 4). The animal may die even at this stage but if it survives, the enlargement continues to reproduce the original bulk of the liver and beyond this to genuine hyperplasia.

Hepatectomy performed five weeks after the commencement of carcinogen feeding leads to similar results if the administration is continued after operation. After seven weeks of feeding however the mortality is much less, and the animals

remain healthier. Liver regeneration is still slow in such animals, but the organ weight reaches the lower limits of normal in some by the fourteenth day. Microscopically such animals show less destruction after operation and a more rapid onset of the nodular regeneration, otherwise similar in type. At the other extreme, if hepatectomy is performed on the day on which feeding with 2-acetylaminofluorene is started there is little delay in the onset of a normal general regenerative reaction, and the liver weight returns to near the normal limits in seven days. There is however the tendency for the weight to remain a little below normal, and nodules start to form as in the other cases. By the fourteenth day such nodules containing many mitotic figures are prominent. Regeneration in animals given three weeks treatment with carcinogen and then two weeks on a normal diet before hepatectomy behave much like those given no treatment before operation. This suggests that an extreme degree of liver damage, likely to cause death after hepatectomy, is due to cumulative toxic action on the liver cells by the 2-acetylaminofluorene. Such an extreme degree of damage is not a prerequisite for the onset of nodular proliferation, although this process is undoubtedly more vigorous in animals threatened with imminent decease from liver failure. It does appear that some degree of "strain" precipitated by functional incapacity, is needed to start this process going. How such a "strain" can be translated into a stimulus which results in the production of a new race of liver cells capable of resisting the toxic effects of 2-acetylaminofluorene, and of still responding to the physiological stimulus for regeneration, remains to be investigated.

So many of the changes which accompany carcinogenesis in any organ must be incidental that to establish a connection between such events and the process of carcinogenesis itself it is essential to show that they do affect the actual production of tumours. In the case under consideration the performance of a hepatectomy at the appropriate period might be expected to alter the latent period or the incidence of liver tumours. It might also shorten the period of administration of carcinogen needed to produce tumours, if the precocious appearance of the changes described, means that the "initial, essential step" (Laird and Barton, 1959), has already been accomplished at the end of three or four weeks. Experiments already carried out by the author (Laws, 1956) have established an effect of hepatectomy, carried out at the beginning of the carcinogen feeding period, on the latent period of liver tumour production, although the incidence of tumours was unaltered. In the first experiment (Table I), animals treated by this pro-

TABLE I

Group	Hepatectomy performed	Hepatoma incidence in periods (after start of AAF feeding) (a) 12–14 months	(b) 15–17 months	(c) 18–24 months	Total
A	None (normal controls)	1	6	1	8/11
B	3 weeks before AAF feeding started (Hepatect. controls)	0	5	3	8/10
C	At start of AAF feeding	5	4	1	10/10
D	After 3 months of AAF feeding	0	1	8	9/11
E	At end of AAF feeding (4 months)	1	3	4	8/10

(Total out of number alive at twelve months.)

cedure showed five deaths with gross liver tumours out of ten animals at a time when only two out of forty-two other rats not hepatectomised early in the feeding period had developed tumours. These other animals included, as well as normal controls, animals hepatectomised after three and four months of carcinogen feeding, that is to say after the onset of hyperplasia. In these latter animals the onset of tumours was, if anything, delayed beyond the period seen in the normals. Skoryna and Webster (1951) also concluded that partial hepatectomies started in the third month of 2-acetylaminofluorene feeding, even if repeated, did not accelerate the appearance of tumours. A further experiment on these lines is still in progress ; the first results are similar to those of the earlier one.

The period by which the appearance of tumours was accelerated was about three months, which coincides roughly with the period taken in our animals for the appearance of hyperplasia on plain 2-acetylaminofluorene feeding. This would fit in with the suggestion of Laird and Barton (1959) that it is from the onset of hyperplasia that the irreversible process of tumour formation should be dated. It would seem that the latent period before the onset of rapid tumour growth is unaffected by hepatectomy outside the " critical " period, which lasts up to the time of onset of hyperplasia during 2-acetylaminofluorene feeding. The finding of Glinos, Bucher and Aub (1951) that in rats treated with dimethylaminobenzene, hepatectomy at the time of withdrawal of the carcinogen appeared slightly to hasten the appearance of tumours, suggests that the mechanism of action of the azo-dye carcinogens may differ from that of 2-acetylaminofluorene. This is made more probable by the difference in the time-course of liver hyperplasia noted by Laird and Barton when using a carcinogen of the azo-dye group (3′-methyl-dimethylaminoazobenzene).

These authors also note that the onset of hyperplasia occurred later in rats which were of greater weight, and incidentally older, at the time carcinogen feeding was started. In another experiment carried out by the author in this laboratory it has been shown that the latent period before the onset of active tumour growth is also affected by the age of the rats at the time at which feeding with carcinogen is started. Two groups of rats were given the same course of four months feeding with 2-acetylaminofluorene. The first group had an average body weight of 100 g. and the second an average of 380 g. at the start. The small rats gained weight during the four months in spite of treatment, the larger animals lost weight. The results, not previously reported, show a significantly earlier incidence of tumours in the young group (Table II). By fourteen months there had been six

EXPLANATION OF PLATE

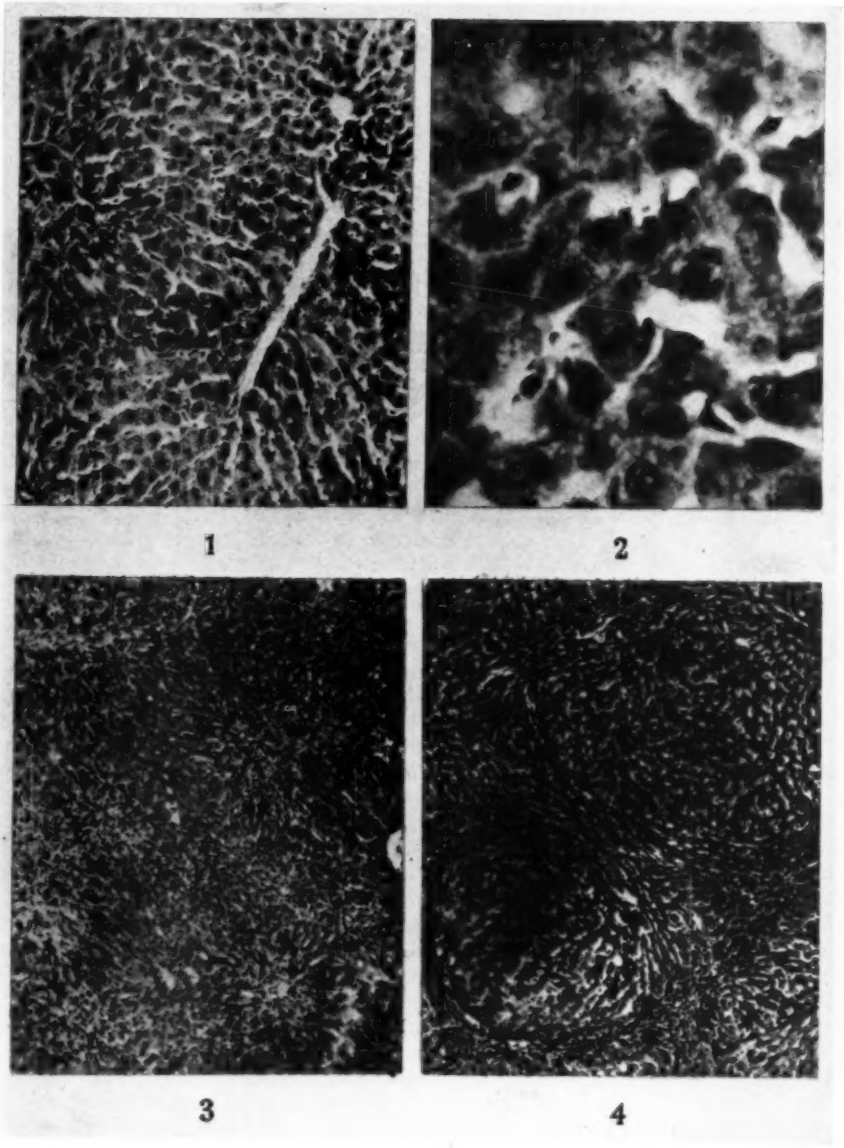
FIG. 1.—Rat 1. Wt. 260 g. Wt. of liver at death 4·5 g. (normal 10 g.). Killed 8 days after partial hepatectomy. Infiltration of cells round portal tracts and basophilic nodules near central veins. × 110.

FIG. 2.—Rat 1. High power view of basophilic nodule with mitosis. × 550.

FIG. 3.—Rat 2. Wt. 260 g. Wt. of liver at death 2·75 g. (normal 10 g.). Found dying nine days after partial hepatectomy. Severe infiltration round portal areas with diminution of parenchyma. Basophilic nodules only beginning to form. × 65.

FIG. 4.—Rat 3. Wt. 360 g. Wt. of liver at death 3·5 g. (normal 13 g.). Found dying 14 days after partial hepatectomy. Liver contained many pale or translucent areas on naked-eye examination. Nodules have enlarged and are compressing the surrounding original tissue and the infiltrating cells. × 65.

(All animals received carcinogen treatment for three weeks before hepatectomy, and subsequently until death.)



1 2

3 4

Laws.

TABLE II

Group	Hepatoma incidence in periods (after start of AAF feeding) (a) 12–14 months	(b) 15–16 months	(c) 17 months and over	Total
Young (100 g. body wt.)	6/7 (i.e. 1 death without hepatoma)	0/1	2/2	8/10
Old (380 g. body wt.)	1/4 (i.e. 3 deaths without hepatoma)	0/3	3/4	4/11

(Hepatoma deaths given as fraction of total deaths.)

deaths with large liver tumours in this group, but only one in the older animals. Later tumours appeared more frequently in the older group. Comparison of the total tumour incidence in the two groups is unsatisfactory since many of the older animals died before reaching the period of highest tumour incidence in this group. There was a difference of about ten weeks in the median tumour death ages for the two groups, which is similar to the difference in the time of onset of hyperplasia noted by Laird and Barton in their experiments with groups of rats of different initial weights, using this carcinogen.

DISCUSSION

Following partial hepatectomy in rats which have received 2-acetyl-aminofluorene, mitotic activity occurs only in the hyperplastic nodules which then appear. Even when the nodules are still small, mitoses have not been seen in the sickly parenchyma around them. Once a nodule has formed mitosis occurs rapidly, and the eventual hyperplastic liver has apparently been formed from the descendants of the comparatively small number of cells which have originated the nodules. This process contrasts with the diffuse mitotic activity seen in normal regeneration, and which is reported by Laird and Barton (1959) as occurring in rats treated with 3-methyl-dimethylaminoazobenzene. For this reason it would seem unwise to compare directly results obtained with the two carcinogens, or to assume that quantitative results obtained with one can be applied to a consideration of the mode of action of the other. Under the conditions of nodular replacement obtaining in the 2-acetylaminofluorene treated animals, regenerative activity is of course slower. The time taken in the experiments for the return of the liver weight to normal levels is sufficiently slow to allow for the extra generations of cells which must be produced. Observations on the similar, though more gradual formation which occurs in uncomplicated carcinogen feeding suggests that here too there is a gradual replacement of " normal " liver cells by the descendants of a small number of cells which have been changed in some way by exposure to the carcinogen. In particular, as suggested by Skoryna and Webster (1951), they appear to have become resistant to the toxic action. In spite of this suggestion that all the cells in a hyperplastic liver are " changed ", tumours arise from only a minority of the cells by a further, later burst of mitotic activity accompanied by other changes in behaviour. It seems that as Laird

and Barton (1959) suggest, an essential step for carcinogenesis occurs when hyperplasia supervenes. With 2-acetylaminofluorene further alterations, perhaps not dependent on the direct action of the carcinogen, are necessary before true tumour formation can occur, as well as subsequently. Further the possibility must be considered that the change in certain cells which results in a resistance to the toxic action of the carcinogen and which enables hyperplasia to occur, may not necessarily be the essential carcinogenic change. It may accompany this, or set the stage for it. Experiments at present in progress on the behaviour of cells isolated from hyperplastic livers into tissue culture may help to throw some further light on the changes which occur at this stage, as may others in which a short period of carcinogen feeding, too short to give rise to tumours normally, is being combined with hepatectomy at the second or third week of feeding.

SUMMARY

(1) Liver regeneration in rats following hepatectomy is severely delayed and disorganised when the carcinogen 2-acetylaminofluorene is administered for three weeks before the operation and subsequently. Regeneration under these circumstances occurs by nodular proliferation from a relatively small number of centres.

(2) When hepatectomy is performed at the beginning of the carcinogen feeding period, the initial phase of regeneration proceeds normally but is quickly superseded by nodular replacement. Such animals show a reduction in the latent period for liver carcinogenesis.

(3) Rats started on a course of 2-acetylaminofluorene treatment at the age of eight weeks also show a reduced latent period for liver carcinogenesis as compared with rats which are adult at the start of the treatment.

(4) It is suggested that during such treatment with 2-acetylaminofluorene an irreversible step in the process of carcinogenesis occurs at the onset of nodular hyperplasia. Hepatectomy early in the feeding period probably brings this step about precociously.

It is a pleasure to acknowledge the technical assistance of Miss Sallie Yates, and the help of Mr. C. Eastcott with the photomicrographs.

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THE EFFECT OF TISSUE OXYGEN TENSION ON THE RADIOSENSITIVITY OF LEUKAEMIA CELLS IRRADIATED *IN SITU* IN THE LIVERS OF LEUKAEMIC MICE

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Received for publication August 29, 1959

A DETAILED description of the lymphocytic type of leukaemia of CBA mice used in the present study, including an assay method for determining the density of viable leukaemia cells in single-cell suspensions prepared from the infiltrated livers of fully leukaemic mice, was given in a previous paper (Hewitt, 1958). The assay method was later used to determine a survival curve for the liver leukaemia cells irradiated *in vivo* in leukaemic mice breathing air during total-body irradiation (Hewitt and Wilson, 1959). Under these conditions a linear relationship was demonstrated between whole-body radiation dose and log survival ratio among the viable leukaemia cells. The D_0 value given by the linear part of the curve (the increment in dose of radiation required to reduce the number of viable leukaemia cells to 37 per cent) was 165 r ^{60}Co gamma radiation. The disposition of the points suggested a 2-hit curve but this feature was not determined with certainty. Comparison of the D_0 value for mouse leukaemia cells with the D_0 value obtained for human HeLa cells irradiated *in vitro* under well oxygenated conditions (Puck and Marcus, 1956) could not be usefully made without information concerning the oxygen tension in the environment of the mouse leukaemia cells at the time of their irradiation *in vivo* in mice breathing air. Comparison of the radiosensitivities of the HeLa cells and mouse leukaemia cells, as described by the established D_0 values, would only be valid if it could be shown that the leukaemia cells were, like the HeLa cells, in a well-oxygenated environment at the time of their irradiation. The importance of this comparison relates to the appropriateness of extrapolation to human tumours of radiobiological data obtained for mouse tumours.

The macroscopic and histological appearances of the livers of the fully leukaemic mice used for determination of the *in vivo* survival curve already referred to suggested that the vascularity of the liver is impaired at this stage of the disease ; and it was considered that an unknown proportion of the masses of vigorously metabolising leukaemia cells infiltrating the liver might have been under severely hypoxic, if not anoxic, conditions at the time of irradiation. If this proportion were large, a rise in the radiosensitivity of the cells might be expected if the mice were exposed to radiation while breathing oxygen instead of air ; on the other hand, rendering the cells anoxic during irradiation, by killing the mouse before exposure, would not be expected to reduce the radiosensitivity significantly. Investigations into these questions form the subject of the present paper.

MATERIALS AND METHODS

Mice.—CBA male mice bred in this laboratory by brother-to-sister mating were used in all the experiments; the mice were 2–4 months old at the time of experiment. The leukaemic mice which were irradiated had been injected intraperitoneally with several million leukaemia cells 9–11 days previously. At the time of irradiation the mice were moderately sick, almost all organs being heavily infiltrated with leukaemia cells.

Irradiation of mice.—The leukaemic mouse was placed in a "Perspex" cylinder of such dimensions as permitted the mouse to assume a normal uncramped resting posture but prevented it from turning round. The cylinder was closed at each end with a rubber bung perforated by a short length of glass tubing, one end of which served as an exit for the gas mixture to be circulated through the cylinder. The gas mixture was allowed to flow in at the opposite end via a flow meter from a cylinder containing the desired gas mixture (British Oxygen Company, Ltd.). Two gas mixtures were used: air containing 5 per cent carbon dioxide, and oxygen containing 5 per cent carbon dioxide. The carbon dioxide was included to ensure an adequate respiratory stimulus. Each gas mixture was allowed to flow through the cylinder at a rate of 1·8–2·2 litres/min. for 10 minutes before, and throughout, irradiation. The "Perspex" cylinder containing the mouse to be irradiated was positioned in a beam of ^{60}Co gamma radiation from a Kilocurie beam unit (a "Theratron"). The whole-body dose was delivered at a mean dose rate of 70–74 r/min. and was given as equal exposures to both sides of the cylinder. The distance from the source to the centre of the cylinder was 62 cm. and the field size used was such as to cover the mouse very generously. Under these conditions, the whole-body dose was uniform throughout the mouse to about $\pm$3 per cent.

At each radiation dose level used, an air-breathing and an oxygen-breathing mouse, both at the same advanced stage of the disease, were exposed separately but on the same day, the two mice being treated under identical conditions except for the different gas mixtures respired. Experiments at different dose levels were done on different days, but mice at a similar stage of the disease were used on all occasions.

For irradiation of the leukaemia cells under what are assumed to be anoxic conditions leukaemic mice were killed by fracture of the neck 1 minute before the start of their exposure to radiation under the same conditions as the living mice. The series of dead mouse experiments was undertaken at a slightly later stage of the leukaemia's history than the living mouse experiments. However, the radiosensitivity of the cells in living mice was determined again after completion of the dead mouse experiments, and was found to be unchanged.

Measurement of the survival ratios in irradiated leukaemia cell populations.—Details of the method of preparing single-cell suspensions of leukaemia cells from the livers of leukaemic mice have been described previously (Hewitt, 1958). In the present experiments, such suspensions were prepared from the livers of the irradiated mice within 20 minutes of the end of their exposure. The density of morphologically intact, and *apparently* viable, leukaemia cells was determined by counting in a haemocytometer by phase-contrast microscopy. 0·2 ml. volumes of serial tenfold dilutions of the counted suspension in 5 per cent CBA mouse serum in Tyrode solution were injected intraperitoneally into groups of

6 CBA male mice. The range of mean cell doses injected was preselected to cover the expected end-point of an assay. The injected mice were observed for a period of 90 days (a period twice as long as the longest latent period ever observed in a mouse injected with a small inoculum of cells of this strain of leukaemia), and the incidence of leukaemic deaths was recorded for each group. From the results, the number of morphologically intact leukaemia cells required to transfer leukaemia to half a group of injected mice was calculated by the method of Reed and Muench (1938). It was found that the yield of morphologically intact leukaemia cells obtained from the livers of irradiated mice within one hour of irradiation was not reduced below that expected from untreated leukaemic mice. The TD50 values obtained for morphologically intact cells from irradiated mice, however were significantly higher than the average value given by cells from untreated mice and were a function of the dose of radiation. Thus, irradiation abolished the reproductive integrity of a proportion of the leukaemia cells without producing immediate morphological changes appreciable by phase-contrast microscopy. It was found previously (Hewitt, 1958) that in 6 assays of leukaemia cells from unirradiated leukaemic mice, the TD50 values varied from 0·7 to 3·0 cells, averaging 2·0 cells. The log survival ratio in an irradiated leukaemia cell population was calculated simply by subtracting the log TD50 given by the irradiated cell population from the log of the average TD50 given by unirradiated populations.

RESULTS

Irradiation of leukaemia cells in mice breathing 95 *per cent air or* 95 *per cent oxygen*

The log survival ratio among the liver leukaemia cells was determined for mice breathing oxygen containing 5 per cent carbon dioxide during irradiation with 800, 1400 or 2000 r total-body radiation. At each dose level the log survival ratio was similarly determined for the leukaemia cells irradiated in a mouse breathing air containing 5 per cent carbon dioxide. The results are recorded in Table I. In Fig. 1, the result of each experiment has been entered in the graph relating log survival ratio and radiation dose. The points obtained are seen in relation to the log survival curve previously obtained for the leukaemia cells irradiated in mice breathing air alone (Hewitt and Wilson, 1959). None of the points departs significantly from the log survival curve previously obtained, and there is no significant difference at each dose level between the survival ratios obtained for cells irradiated in mice breathing 95 per cent air and for cells irradiated in mice breathing 95 per cent oxygen.

TABLE I.—*Log Survival Ratios among Leukaemia Cells Irradiated* In Vivo *in the Livers of Leukaemic Mice Breathing* (a) 95 *per cent Oxygen*, (b) 95 *per cent Air, during Irradiation*

Dose of radiation (r; ^{60}Co gamma rays)	Log survival ratio	
	Mice breathing oxygen	Mice breathing air
800	$\bar{3}\cdot83$	$\bar{3}\cdot72$
1400	$\bar{4}\cdot55$	$\bar{4}\cdot90$
2000	$\bar{5}\cdot20$	$\bar{6}\cdot53$

Irradiation of leukaemia cells in mice immediately after death

It will be appreciated that in the dead mouse experiments the leukaemia cells are allowed to remain in the livers of the dead mice for a period slightly longer than the length of time occupied by the exposure to radiation, and that during this time they would be expected to be under strictly anaerobic conditions and at a temperature falling gradually from 37° C. to room temperature. It was conceivable that a proportion of the cells might lose their viability under these

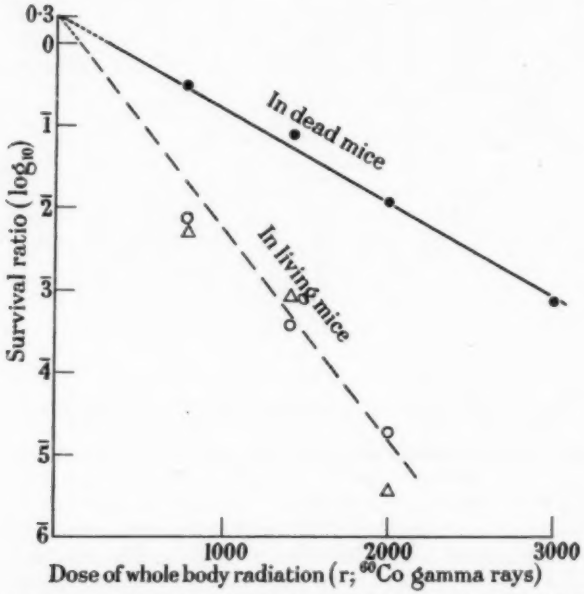

FIG. 1.—Survival curves for leukaemia cells irradiated (*a*) in dead mice, (*b*) in living mice.
– – – – – Survival curve for cells irradiated in living mice breathing air (Hewitt and Wilson, 1959).
○ Survival ratios for cells irradiated in mice breathing 5 per cent CO_2 in oxygen.
△ Survival ratios for cells irradiated in mice breathing 5 per cent CO_2 in air.

conditions. If this were so, the viability loss for cells irradiated in dead mice would be the summation of loss due to anoxia and starvation, and loss due to radiation-induced damage. The longest time of irradiation in these experiments was under 43 minutes (when 3000 r was delivered at a rate of 70·6 r/min.). A preliminary experiment was therefore done in an attempt to detect a rise in the TD50 value given by the leukaemia cells after their residence in the liver of a dead leukaemic mouse at room temperature for 47 minutes after death. A portion of liver was removed from a leukaemic mouse immediately after death by neck fracture and the operation wound was sewn up and the mouse allowed to remain at room temperature. The TD50 of the leukaemia cells released from the excised fragment was then determined. A second liver sample was removed from the mouse 47 minutes after death and the TD50 determined for

the cells in this fragment. The TD50 values obtained were 10 cells and 3·2 cells respectively. It is concluded that the viability loss detected among the cells irradiated in dead mice was due to radiation-induced damage alone and was not contributed to by environmental influences associated with temporary residence of the cells in the tissues of a dead mouse.

TABLE II.—*Log Survival Ratios Among Leukaemia Cells Irradiated in the Livers of Leukaemic Mice soon after Death*

Dose of radiation (r; ^{60}Co gamma rays)	Log survival ratio
800	$\bar{1}$·44
1400	$\bar{2}$·83
2000	$\bar{3}$·98
3000	$\bar{4}$·79

The log survival ratios obtained for leukaemia cells irradiated in dead mice are recorded in Table II, and it is seen from the upper curve of Fig. 1 that there is, again, a linear relationship between log survival ratio and the dose of radiation. From the linear part of the curve, which extrapolates to cut the zero dose axis at about +0·3, the D_0 value is approximately 380 r, compared with 165 r for the leukaemia cells irradiated *in vivo* in mice breathing air or oxygen. Thus, for equal survival ratios, the dose required when the cells are under what are assumed to be anoxic conditions is greater than that required when the cells are irradiated in what is assumed to be a moderately well-oxygenated environment, by a factor 2·3 approximately.

Theoretical radiation survival curves for leukaemia cell populations consisting of known proportions of anoxic and well oxygenated cells

The linearity of the log survival curves for both anoxic and well-oxygenated leukaemia cells suggests that in each case the cells of the exposed population were remarkably uniform in respect of their environmental oxygen tension. For the cells in dead mice such uniformity is to be expected, since it is inconceivable that foci containing available oxygen could persist among rapidly metabolising cells within an organ whose circulation has ceased. For the cells in mice breathing oxygen or air the apparent uniformity is more surprising: we should expect a proportion of the cells to lie in situations where thrombosis or other vascular accident has given rise to virtually anoxic foci. Areas resembling infarcts, in which both the liver cells and the infiltrating leukaemia cells have undergone necrosis, are indeed to be seen occasionally in advanced leukaemic livers. In the case of many solid tumours, which show extensive areas of necrosis in histological section, it cannot be doubted that many of the malignant cells at the boundary of necrotic zones would be under anoxic conditions. Since it appears probable that the cells of many tumours are heterogeneous in respect of the oxygen tension in their environment it is useful to consider the character of theoretical log survival curves for cell populations consisting mostly of well-oxygenated cells but containing a known proportion of anoxic cells, each variety of cell having a radiosensitivity defined by the appropriate D_0 value as determined here for

well-oxygenated and anoxic leukaemia cells. It is reasonable to assume that the respective radiosensitivities would not be influenced by the fact that the cells belonged to a mixed population, so that the survival ratio for the total population after any dose of radiation can be expressed as follows :

$$\frac{\text{Surviving well-oxygenated cells} + \text{surviving anoxic cells}}{\text{total initial cell population}}.$$

With increasing doses of radiation, the viable anoxic cells, being eliminated at a slower rate than the well-oxygenated cells, will form a rapidly increasing

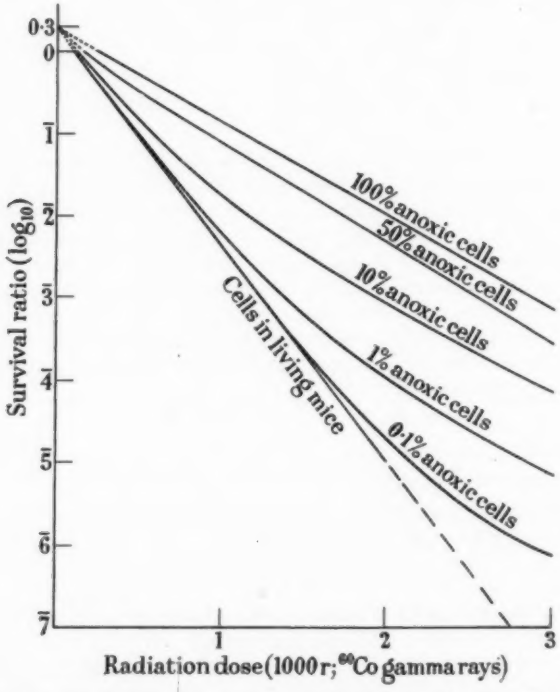

FIG. 2.—Theoretical radiation survival curves for leukaemia cell populations consisting of mixtures of well-oxygenated and anoxic cells.

proportion of the total surviving population of viable cells. The log survival curve for the total population will thus gradually assume the slope for a pure population of anoxic cells. For example, after exposure to 2500 r of an initial mixed population of 10^6 cells, consisting of 10 per cent anoxic cells and 90 per cent well-oxygenated cells, there is less than a 50 per cent chance of one viable well-oxygenated cell surviving, whereas about 250 viable anoxic cells would still remain. In Fig. 2 the original separate linear log survival curves for pure populations of

well-oxygenated and anoxic leukaemia cells, respectively, are shown. Between these, are shown theoretical curves for mixed populations containing various stated proportions of the two types of cell. It will be seen that populations containing only a small proportion of anoxic cells give a log survival curve slope which does not depart significantly from that for well-oxygenated cells until higher doses of radiation are attained, when the slope changes gradually to that for anoxic cells.

DISCUSSION

A positive correlation between radiosensitivity and environmental oxygen tension has been demonstrated for a wide variety of cells, including several mammalian tumours (Gray, 1957); the observed ratio of the radiosensitivities of anoxic and moderately well-oxygenated normal tissue cells has been similar to that recorded here for leukaemia cells. For example, Howard-Flanders and Wright (1957), using a quite different indicator—the inhibitory effect on bone growth in the mouse tail, found relative radiosensitivity values of 1, 1·97 and 2·56 respectively for the anoxic tail (with occluded blood supply) and the tail in air-breathing and in oxygen-breathing mice. Using visible chromosome damage as an index of radiosensitivity for Ehrlich ascites tumour cells irradiated with X-rays at 18° C. *in vitro*, Deschner and Gray (1959) showed that the relative radiosensitivity of the cells rose rapidly from a minimum value of 1·0 for anoxic cells to about 2·3 for cells in fluid equilibrated with oxygen at a pressure of 20 mm. Hg. With oxygen tensions above this level, radiosensitivity increased more gradually, a value of 3·0 not being attained until the oxygen pressure reached about 400 mm. There is no reason to believe that a similar relationship between oxygen tension and radiosensitivity does not obtain for mammalian tumour cells irradiated *in vivo*, although the environmental oxygen tension of tumour cells *in vivo* would not be expected to be uniform and would not be measurable with the precision possible with an *in vitro* system. Nevertheless, the results with mouse ascites cells (Deschner and Gray, 1959) and other results with bacteria (Alper and Howard-Flanders, 1956) make it probable that the range of oxygen tensions over which we should expect major alteration of the radiosensitivity of mouse leukaemia cells *in vivo* is from zero to about 20 mm. Hg. The range with which we are concerned thus lies distinctly below the tension (40 mm.) normally found in the veins of an air-breathing mammal.

The local tissue oxygen tension for any small group of tumour cells *in vivo* cannot at present be ascertained by direct measurement, although it is possible to calculate theoretical values from various assumptions and data. Such values have been calculated (Thomlinson and Gray, 1955) for foci within squamous carcinomas of human lung, and have been strikingly correlated with the actual spatial relationships of necrotic foci and capillaries as seen in histological sections of these tumours. The complexity of the factors influencing the oxygen tension in the vicinity of tumour cells *in vivo*, and the effects of raising the partial pressure of oxygen respired have been discussed in great detail by Churchill-Davidson, Sanger and Thomlinson (1957). These considerations cannot, however, provide a reliable assessment of the proportion of viable tumour cells which are actually anoxic in man or animals breathing oxygen at normal or supranormal pressures. The theoretical curves shown in Fig. 2 suggest that the proportion of mouse

leukaemia cells in the livers of leukaemic mice breathing air or oxygen which are anoxic, is certainly less than 1 per cent and possibly no greater than 0·1 per cent. The proportion of anoxic cells in other tumours, those showing more widespread vascular disturbance, may very well prove to be greater.

It is clear that the relative radioresistance of anoxic tumour cells is such that the presence of these cells in a tumour *in vivo* would be expected substantially to diminish the effectiveness of tumour radiotherapy. It is, therefore, important to discuss certain theoretical considerations concerning the possible incidence of anoxic tumour cells *in vivo*. It is certain that vascular occlusion frequently leads to death of cells, often involving quite large volumes of tumour tissue. Such large-volume necrosis supposedly results from total deprivation of the metabolic requirements of the cells, including glucose, amino acids, vitamins and other growth requirements, as well as oxygen; the accumulation of waste products also may contribute to the necrosis. The predicament of such grossly deprived cells is sooner or later lethal, and their temporary survival in a tumour would have no influence on its radiocurability. The cells whose relative radioresistance would be of importance to radiocurability are those which are almost or actually anoxic but which nevertheless have their viability preserved over the period of time required for them to reproduce over several generations. This situation implies a differential interference with cell requirements, such that adequate amounts of glucose and other growth factors continue to be supplied, while available oxygen falls very severely. We do not know whether the tissue fluid commonly attains a composition which permits these conditions to prevail, and it is clear that more information is required before it can be assumed that groups of tumour cells may pass through a fairly prolonged period of severe hypoxia *in vivo* and later assert themselves as the progenitors of a massive tumour cell population. Our results suggest that such anoxic cells are uncommon, even in the heavily infiltrated leukaemic mouse liver, where anoxic conditions might be expected.

Although our results suggest that anoxic cells are unlikely to form more than a small proportion of the total malignant cell population in an air- or oxygen-breathing mouse, it should be appreciated that even a very small proportion of such cells could very significantly affect the dose of radiation required to eliminate the growth potential of a large population of tumour cells. A tumour 2 cm. in diameter and consisting half of stroma and half of tumour cells (mean diameter 12·6 μ), with only half the tumour cells capable of reproduction, would contain about 10^9 reproductively intact malignant cells. Now if each of these cells is capable of regenerating a fresh tumour, a survival ratio of about 10^{-10} is required for a 90 per cent chance of eliminating the total malignant cell population. In Fig. 3, the linear survival curves obtained for leukaemia cells irradiated under well-oxygenated and anoxic conditions, respectively, have been extrapolated to very low survival rates. It will be seen that a survival ratio of 10^{-10} would be expected after exposure of the malignant cell population to a ^{60}Co gamma radiation dose of 4000 r, provided that all the cells were under well-oxygenated conditions at the time of irradiation. If, however, 10^5 (0·01 per cent) of the cells were under anoxic conditions during exposure, the curve for anoxic cells indicates that after exposure to 4000 r there would be about a 90 per cent chance of one or more reproductively intact cells surviving the irradiation. 10^5 cells, of the size given, occupy a volume of only 0·1 c.mm. This very small volume of anoxic

cells, present in a tumour of 2 cm. diameter, would thus seriously militate against eradication of the tumour by this dose of radiation, and be responsible for reducing an expected 90 per cent cure rate to about 10 per cent. If all the tumour cells were anoxic during irradiation, as would be the case if the vascular supply of the tumour were to be totally interrupted by pressure or torsion, then about 9000 r would have to be delivered to the tumour before a useful cure rate could be expected.

The D_0 value obtained by Puck and Marcus (1956) for human HeLa cells irradiated *in vitro* under well-oxygenated conditions with 230 kV X-rays, was 96 r. Puck, Morkovin, Marcus and Cieciura (1957) found a similar value for

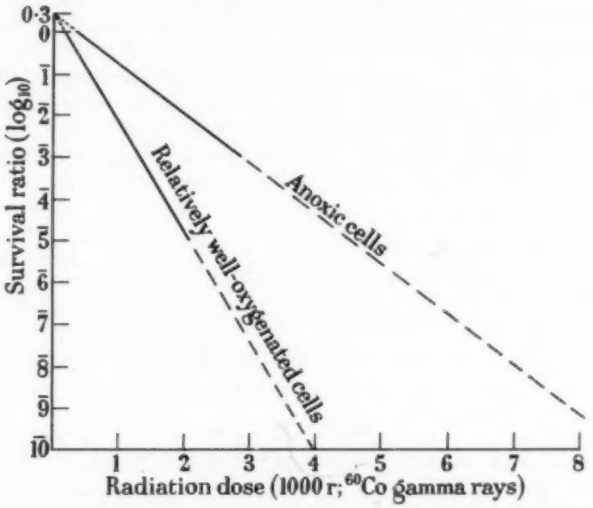

FIG. 3.—Extrapolated survival curves for leukaemia cells irradiated under well-oxygenated conditions (in air-breathing mice), and under anoxic conditions (in dead mice).

numerous other human epithelial cell types under similar conditions. Recently Morkovin, and Feldman (1959) pointed out that an error in the original dosimetry requires the value of 96 r to be increased by a factor 1·45 to give an adjusted D_0 value of 139 rads. When further increased by the factor 1·25 to allow for the greater R.B.E. of 230 kV X-rays compared with ^{60}Co gamma rays, the D_0 value for human epithelial cells irradiated under well-oxygenated conditions becomes 174 rads of ^{60}Co gamma radiation, which is not significantly different from the D_0 value (161 rads) obtained here for murine leukaemia cells irradiated *in vivo* in air-breathing mice. This remarkably good correlation between the radiosensitivities of human and murine cells suggests that parameters obtained from radio-biological studies of mouse tumours may be directly applicable within the sphere of clinical radiotherapy of tumours. It may be added that consideration of the implications of such parameters should properly precede the use of such procedures as the treatment of human leukaemia by whole-body radiation.

SUMMARY

A transplantation bio-assay method was used to determine survival ratios among the leukaemia cells released from the livers of leukaemic mice immediately after their exposure to 800, 1400 and 2000 r total-body ^{60}Co gamma radiation, a surviving cell being defined as one capable of securing successful transplantation of the leukaemia. No significant difference was demonstrated between the survival ratios obtained for cells from mice breathing 95 per cent oxygen and from mice breathing 95 per cent air during irradiation. None of the survival ratios departed significantly from the linear log survival ratio-radiation dose curve obtained previously for leukaemia cells irradiated in mice breathing air (Hewitt and Wilson, 1959). A linear relationship was demonstrated between log survival ratio and radiation dose for the leukaemia cells irradiated under anoxic conditions (in recently killed mice). Comparison of the log survival curves for cells irradiated in mice breathing air or oxygen and for the anoxic cells showed that the latter were more radioresistant by a factor 2·3. The slope of the log survival curve for cells irradiated in living mice was closely similar to that obtained by Puck and Marcus (1956) for human cancer cells (HeLa) irradiated under well-oxygenated conditions *in vitro*.

We are indebted to Miss J. Howarth, B.Sc. (B.E.C.C. Research Physicist) for carrying out all the irradiations done using the "Theratron", and to Mrs. D. Levy and Miss E. Blake for skilled technical assistance with the biological work. We are grateful to the British Empire Cancer Campaign for a whole-time grant to one of us (H. B. H.) and for financial support to the laboratory of the other (C. W. W.).

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QUANTITATIVE STUDIES WITH MH2 RETICULO ENDOTHELIOMA VIRUS

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Received for publication September 18, 1959

THE chorioallantoic membrane (CAM) of a developing chick embryo has been extensively used for the study of the quantitative aspects of the multiplication of Rous Sarcoma virus. Rous and Murphy (1912) first demonstrated that when the Rous Sarcoma I virus was inoculated into the embryonic membranes of a chick embryo tumours developed at the site of inoculation. Keogh (1938) inoculated Rous Sarcoma virus extract on the intact ectodermal surface of the CAM and produced ectodermal lesions or pocks. The quantitative aspects of titrating Rous Sarcoma virus extracts on CAM has been followed up by Rubin (1955), Prince (1958*a*, *b*, *c*) and Vigier (1959). Unlike Keogh, these workers did not experience the large range of variation between eggs which had led to the neglect of assaying these viruses on CAM.

The present study deals with some aspects of the quantitative titration of MH2 reticulo endothelioma virus (Murray and Begg, 1930), especially on the CAM.

MATERIALS AND METHODS

Preparation of virus extract

Crude extracts were prepared by grinding tumours from chicks or infected CAMs in 10 volumes of sterile water in glass homogenisers. The cells and debris were removed by centrifuging at 1000 g. for 10 minutes.

Purified preparations of virus according to the method of Bather (1953) were used in a few of the parallel titrations.

Titration of virus

Embryos and day old chicks used for titration were obtained from the inbred flock of Brown Leghorns selected for susceptibility to Rous sarcoma virus by Dr. Carr and maintained at the Poultry Research Centre.

The extracts were titrated on the CAM of chick embryos according to the method of Keogh (1938). Embryos of various ages (6–12 days old) were used. A triangular window was drilled very carefully to minimize damage to the CAM and the CAM was dropped in the usual manner. 0·1 c.c. of extract was inoculated on the dropped CAM and the window was sealed with Scotch tape. The embryos were then incubated at 38° C. for 7 days. The eggs were candled daily to detect dead embryos. To count the pocks, the CAMs were carefully cut out, rinsed in water and spread out on a petri dish which had a grid marked out on its back. The pocks were counted against a dark surface with a tally counter.

Titrations in day old chicks were done according to the method of Carr and Harris (1951). 0·2 c.c. of ten-fold dilutions were inoculated into the right thigh in groups of 3 or 4 day old chicks. The virus titre was calculated according to the method of Parker and Rivers (1936).

RESULTS

Percentage of non reactors

The percentage of non reactors to Rous Sarcoma virus was used by Prince (1958*a*) to select the most susceptible strain of embryos for the CAM assay technique. He found that White Leghorns produced the least percentage of non reactors, only 10 per cent of embryos failing to produce any pocks even when inoculated with very high doses of virus. Vigier (1959) also found non reactors but their number decreased with increase of virus titre.

Table I gives the percentage of non reactors for the different ages of embryos of Brown Leghorns used. To make the results comparable with those of Prince the percentage of non reacting membranes is only calculated from groups of embryos having an average of at least 20 pocks so that distributional zeros may not be included.

TABLE I.—*Relationship Between Percentage of Non Reactors and the Age of Embryos Used*

Age of embryos (days of incubation at 38° C.)	Number of CAMs tested	Percentage of non-reactors
6	44	11·3
7	40	5·0
8	91	5·5
9	63	1·6
10	195	0·5
11	20	0
12	38	0

From Table I it is obvious that the percentage of non reactors is reduced with increasing age of embryos. This would support the hypothesis that non reactors are not due to the presence of neutralising antibody in the blood stream of the embryos (Prince, 1958*b*). It is known that maternal antibody is transmitted to the embryo through the ovary via the yolk (Andrewes, 1939). It has been shown that antibody present in the yolk is released into the circulation of the embryo in greater amounts with increasing age of embryo (Schechtman and Knight, 1955). Thus, if neutralising antibody was the cause of non reactors it would be expected that the percentage of non reactors would increase with the age of embryos.

Effect of age of embryo on pock count

To compare the number of pocks produced by embryos of different ages standard amounts of the same extract were inoculated into groups of embryos of different ages. Eight to twelve embryos were usually inoculated for each age group. Embryos of four different ages, viz. 6, 8, 10 and 12 days old were used for these experiments.

TABLE II.—*Mean Number of Pocks Produced on CAMs After Inoculation of Same Dose of MH2 Virus Extract on Chick Embryos of Different Ages*

Expt. No.	Age of embryos: 6 days — Number of embryos	Mean ± SE	Coeff. of var.	8 days — Number of embryos	Mean ± SE	Coeff. of var.
I	9	18·3±6·3	96·3	11	21·7±7·5	114·6
II	4	26·3	—	4	28·7	—
III	6	5·5±1·2	43·3	8	8·7±4·2	135·5
IV	—	—	—	8	6·7±1·7	74·1
V	—	—	—	12	17·7±4·9	95·9
VI	—	—	—	8	12·4±2·4	54·6

Expt. No.	10 days — Number of embryos	Mean ± SE	Coeff. of var.	12 days — Number of embryos	Mean ± SE	Coeff. of var.
I	12	43·3±5·2	41·1	11	48·9±7·3	49·4
II	6	46·5	—	8	58·6	—
III	8	63·2±7·5	35·3	8	58·2±9·7	40·9
IV	8	32·8±4·5	30·8	—	—	—
V	9	31·5±6·5	58·6	—	—	—
VI	8	42·4±7·1	44·1	—	—	—

The results of the various age experiments are summarized in Table II. Although there is a slight overall increase in the number of pocks associated with an increase in the age of embryos (the only exception being Experiment III where there is a slight decrease in the number of pocks from 10 day to 12 day embryos) the greatest difference is between 8 day and 10 day old embryos. There is some variation between experiments as to the increase in the number of pocks between embryos of these two age groups, but from the means for all the experiments it appears that on the average 10 day old embryos produce 2½ times as many pocks as 8 day old embryos. It was also noted that the pocks in 12 day and 10 day old embryos were slightly larger than those in 8 day and 6 day old embryos. However, there was a large range of variation in the size of pocks within each group.

Comparison between virus titre values from day old chicks and pock counts on CAM

Parallel titrations in day old chicks and on the CAMs of mostly 10 day old embryos are compared graphically in Fig. 1 and summarized in Tables III, IV and V. The details for the various experiments are given below. As seen in Fig. 1 and Tables III, IV and V, the titration values from day old chicks are much higher than the corresponding values from the pock titre on CAM. The last column in Tables III, IV and V gives the log difference between the two titration values. It appears that the CAM assay method is 1 log to 2 log less sensitive than the titrations in day old chicks.

Table III gives the results of three experiments where the virus titres were compared without passage on the CAM (1st transfer generation), and after one passage to see if there was any adaptation. In all the experiments there appears

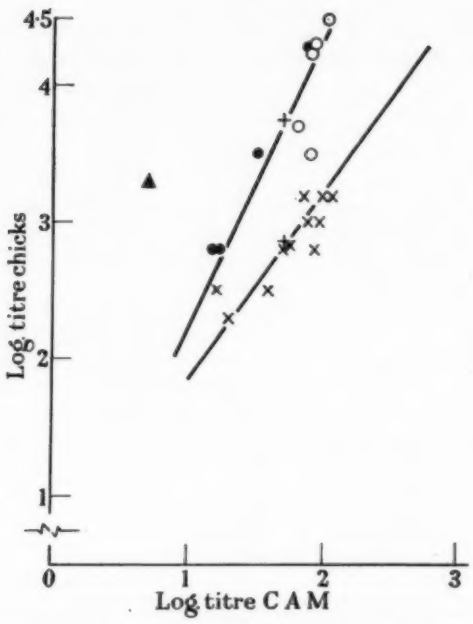

FIG. 1.—Relationship between titres calculated from pocks on CAM and the end point technique in day-old chicks.

Regression (b) for chick-derived virus = 0·45
Regression (b) for CAM-derived virus = 0·72

(1) Difference between the two regression coefficients is not significant ($0·5 > P > 0·1$).
(2) Sample difference in elevation of the two regression lines is highly significant ($0·001 > P$).

Mean of each group indicated by +.

● 1st transfer to CAM.
× Further passages on CAM.
○ Multiple CAM passages followed by 1 or 2 in chicks.
▲ 8-day embryos used.

TABLE III.—*Relationship Between Titration Values from Pock Counts on CAM and Day Old Chicks Without Passage on CAM and After One Passage*

Expt. No.	Transfer generation on CAM	Mean pock count CAM 0·1 c.c.	Mean pock count CAM 0·2 c.c.	Log pock titre CAM 0·2 c.c.	Log virus titre chicks 0·2 c.c.	Log difference
I	1	7·25±0·9	14·5	1·16	2·8	1·64
	2	51·12±7·5	102·24	2·01	3·2	1·19
II	1	2·4 ±0·06	4·8	0·61	3·2	2·62*
	2	7·84±0·54	15·68	1·2	2·5	1·3
III	1	15·77±1·84	31·54	1·5	3·5	2·0
	2	34·71±2·39	69·42	1·84	3·2	1·36

* Use of 8-day-old embryos.

Note : 1st transfer generation on CAM = virus derived from chick tumour without any previous passage on CAM.

TABLE IV.—*Relationship Between Titration Values from Pock Counts on CAM and Day Old Chicks After Passage of Virus on CAM for a number of Transfer Generations*

Expt. No.	Transfer generation on CAM	Mean pock count CAM 0·1 c.c.	Mean pock count CAM 0·2 c.c.	Log pock titre CAM	Log virus titre chicks	Log difference
I	1	9·57±0·97	19·14	1·28	2·8	1·52
	2	47·25±4·28	94·5	1·98	3·2	1·22
	3	42·0 ±5·69	84·0	1·92	3·0	1·08
	4	18·83±2·61	37·66	1·57	2·5	0·92
	5	24·25±1·92	48·5	1·69	2·8	1·11
II	1	36·2 ±4·93	72·4	1·86	4·3	2·44
	4	40·25±2·69	81·7	1·91	2·8	0·89
	5	37·5	75·0	1·88	3·0	1·12
	6	6·71±1·66	13·42	1·28	2·3	1·02
	7	24·5 ±1·75	49·0	1·69	2·8	1·11

Note : 1st transfer generation on CAM = virus derived from chick tumour without any previous passage on CAM.

TABLE V.—*Relationship Between Titration Values from Pock Counts on CAM and Day Old Chicks After a Number of Passages on CAM Followed by a Number of Passages on Chicks*

Details of transfer generation (tg)	Mean pock count CAM 0·1 c.c.	Mean pock count CAM 0·2 c.c.	Log pock titre CAM	Log virus titre chicks	Log difference
5 tg on CAM and 1 tg on chicks	42·4±7·1	84·8	1·93	3·5	1·57
7 tg on CAM and 2 tg on chicks	31·3±5·87	63·6	1·80	3·7	1·9
11 tg on CAM and 1 tg on chicks	57·8±5·68	115·6	2·06	4·5	2·44
7 tg on CAM and 4 tg on chicks	36·0±4·52	72·0	1·86	4·3	2·44
7 tg on CAM and 5 tg on chicks	35·7±3·28	71·4	1·85	4·3	2·45

to be a slight adaptation to the CAM after one passage—the log difference between the two titration values being significantly less after one passage on the CAM. In Experiment II in the 1st generation on CAM there is a greater difference between the two values than in the other experiments due to the use of 8 day old embryos for titration on CAM.

Table IV gives a comparison between titration values after the virus had been propagated for a large number of transfer generations on the CAM to see if any further adaptation would take place. The first transfer generation on CAM indicates that the virus is passed on the CAM for the first time. Continual passage of virus on the CAM does not make any difference to the extent of adaptation, the difference remaining about 1 log.

To see if the adaptation to the CAM is permanent, virus, after a number of transfer generations on CAM, was inoculated into chicks and passed for one or more transfer generations on chicks. Parallel titrations were then carried out on CAM and day old chicks. The results and details of transfer generations are shown in Table V. In Table V the log differences in virus titre after even one passage on chicks are comparable with the log difference shown in Tables III and

IV when the virus had not been passed on the CAM at all. The overlapping of these points can be seen in Figure 1. From this it appears that the slight adaptation obtained after one or more passages on CAM is lost as soon as the virus is returned to chicks.

The difference in log titre when the virus is titrated on the CAM for the first time varies from 1·5 log to as much as 2·5 log in some of the experiments. After one or more passages on CAM, i.e. after adaptation, this difference remains fairly constant around 1 log.

DISCUSSION

The titration of MH2 reticuloendothelioma virus on CAM of chick embryos shows a number of interesting results. The percentage of non reactors in the strain of Brown Leghorns used, after 9 to 12 days of incubation, is well below that obtained by Prince (1958*a*, *b*) using 12 day old embryos even with the most susceptible strain (White Leghorns). Prince (1958*b*) also showed that the difference between the highly resistant strain (Fayoumi) and the highly susceptible strain (White Leghorns) can be explained on a genetic basis. He found that resistance was a genetic trait which was neither sex-linked nor passed by maternal inheritance. His data agreed with the hypothesis that resistance was controlled by a single pair of allelic genes, the allele for sensitivity being dominant to that of resistance.

The extremely low percentage of non reactors obtained can be explained by the fact that the embryos used are derived from a line of birds selected for susceptibility to Rous sarcoma virus over a number of generations. Susceptibility to one type of tumour virus may result in susceptibility to other types of tumour viruses.

The reason for the large increase in the number of pocks between 8 day and 10 day old embryos is unknown. On about the 9th day of incubation the haematopoietic tissue in the peripheral blood of embryos reaches stability (Fennell, 1947). The mean percentage of primitive erythroblasts decreases while that of definitive erythrocytes increases to over 90 per cent from about 10 per cent on the 7th day of incubation. Between 9 and 12 days of incubation Fennell (1947) observed the appearance of thrombocytes in the peripheral circulation of chick embryos. The most striking change after the 10th day is the extensive vascularisation of the CAM. From the 10th day the capillaries begin to push outwards towards the shell membrane so that by the 15th day the CAM is extensively covered by a fine network of capillaries with a few ectodermal cells reaching the surface through the capillary net (Danchakoff, 1917). The increased number of pocks between 8th and 10th day embryos may be associated with the various haematopoietic changes in the peripheral circulation of the embryo. It may be due to the extensive vascularization of the CAM especially as the MH2 is a reticuloendothelioma virus and would tend to infect the walls of blood vessels. The increase in pocks may be connected with the biochemical requirements of the virus correlated with the coming into function of some essential organ in the chick embryo, e.g. between 9 to 12 days the spleen begins to act as a haematopoietic organ (Olson, 1943). It is possible that only variants lacking certain biochemical requirements are able to grow in younger chick embryos.

The CAM assay method has certain advantages over the other titration techniques available for avian tumour viruses. While all the other titration methods are based on end point techniques in fowls and chicks (Claude and Rothen, 1940 ;

Carr and Harris, 1951 ; Bryan, 1955) the CAM titration gives a direct count of the number of infective virus particles. Moreover, the CAM titration gives results in a much shorter period compared with the other techniques.

The difference in the values obtained by titrating in day old chicks and by the pock count technique on CAM is very striking for the MH2 virus. Rubin (1955) using Rous sarcoma virus compared the titre obtained by the pock count technique on the CAM with those obtained by the subcutaneous inoculation of 2 weeks and 8 weeks old chicks. He found the CAM technique to be more sensitive than the 50 per cent end point technique on birds. However, when he compared his results with those of Bryan (unpublished), who assayed the same stable stock of virus, he found that the CAM assay agreed very closely with the average of the ID 50 assays in chickens. Prince (1958*a*) also compared the titres obtained by the CAM technique with those obtained by the wing web assay technique for the same virus. He found no difference between the two titration values. Vigier (1959) also found no difference between pock titre from CAM assay and the end point titre in 1–2 months old chicks for Rous sarcoma virus.

The difference in the titration values by the two methods for the MH2 virus is less likely to be due to virus variants. Compared with the Rous sarcoma virus, a virulent strain, which has been passaged almost continuously for 50 years, the MH2 is of more recent isolation, has been passaged much less (much of its existence being spent as a freeze-dried conserve) and is very much less virulent in older chicks (Carr, personal communication). It is possible that the increased susceptibility of the day old chicks as against the CAM is a continuation of the age effect. The CAM is a short-lived tissue with a life-span of about 19 days and in 10 day old embryos the CAM is already an ageing tissue and hence might be less sensitive to the MH2 virus. On the other hand, the embryo itself is more sensitive to MH2 virus than the day old chick for intravenous titrations of MH2 virus in groups of 14 day old embryos gave an end point which was much higher than that obtained by titrating the same extract in day old chicks (unpublished data).

Passage of the virus on the CAM for one generation produces a slight adaptation to the CAM which is lost as soon as the virus is returned to chicks. The fact that the difference in log titre is more variable before adaptation and becomes more constant after, suggests that the change is due to a selection of a variant. It may be a variant with increased capacity to infect uninjured cells or the adaptation may be due to a selection of an ectodermal specific variant which would be lost as soon as the virus is injected into the muscle of the chick.

It is clear that the results obtained with the Rous sarcoma virus are not applicable to other fowl tumour viruses and that the quantitative techniques evolved for this virus are, in the absence of any other information, best restricted to that virus.

SUMMARY

When MH2 virus is assayed on the chorioallantoic membrane the result depends very largely on the age of the embryo, the pock count on membranes of embryos aged 10 days being $2\frac{1}{2}$ times that on those 8 days old. This was always much less than that determined in the same strain of day old chicks by the limiting dilution method. Slight adaptation of the virus to egg passage was found but this was lost when the virus was transmitted through chicks. Non reactor eggs were

few, and the number decreased markedly with the increasing embryo age, to vanish at age 11 days and over.

This work was done while on a grant from the Government of the Federation of Malaya. I am grateful to Professor C. H. Waddington for laboratory facilities and to Dr. J. G. Carr for valuable advice. I am also grateful for the material and facilities provided by the British Empire Cancer Campaign Unit at the A.R.C. Poultry Research Centre.

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INVESTIGATIONS OF A FACTOR FOUND IN CERTAIN NORMAL TISSUES INHIBITING ASCITES TUMOR GROWTH IN THE RAT AND MOUSE

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Received for publication September 3, 1959

It has long been known to pathologists that apparently normal cancer cells may circulate in the blood stream of cancer patients and eventually lodge in the lungs or other tissues where the great majority are inactivated and do not cause metastases. This may be merely a question of viability and the stroma-inducing ability of the cancer cells, but there is also a possibility that antibodies, or some local intrinsic tissue factor might be responsible. The last possibility has been under investigation by Druckey *et al.* for several years (Druckrey, Schmähl and Rajewsky, 1958 ; Steinhoff, Flaschenträger and Bannasch, 1958 ; Schmähl, Bannasch and Flaschenträger, 1958). Druckrey uses a simplified experimental system, consisting simply of incubating Yoshida ascites cells for several hours with various normal rat tissue homogenates, and then injecting the incubated cells intraperitoneally into rats of the same strain. Druckrey found that several tissue homogenates were responsible for a marked inhibition of tumor growth on reinjection (Druckrey, Schmähl and Rajewsky, 1958) and for microscopic damage to the tumor cells (Steinhoff, Flaschenträger and Bannasch, 1958). The effect was especially prominent with lung and spleen homogenates.

The experiments to be described were designed to elucidate further the nature of this phenomenon and the characteristics of the factor responsible. It was attempted :

(*a*) to verify Druckrey's results on other tumors and species;

(*b*) to eliminate by a series of checks and controls the possibility that the effect was due to physical or chemical properties of the tissue homogenates (pH, osmolarity, electrolyte composition) ;

(*c*) to see whether a tumor normally metastasizing to the lung would still be affected similarly by lung tissue ;

(*d*) to see whether tissue homogenates from tumor-bearing animals act in the same way as those from healthy animals ;

(*e*) to determine which cell fraction or fractions contain the responsible factor.

MATERIALS AND METHODS

Species and tumors

(1) The " G–6 " tumor in Wistar WAG pure bred white rats. This was originally a spontaneous mammary tumor of the rat ; in its solid transplantable form it metastasizes frequently to the lungs. The ascitic form used here had been passed in the Wistar rat for 30 generations and had attained a uniform pattern,

5×10^6 cells injected intraperitoneally regularly killing the animal, without metastases, in 12 to 15 days.

(2) The Ehrlich ascites tumor, in pure bred white mice of Strain A. Intraperitoneal injection of 5×10^6 cells caused the animal's death in about 20 days.

Preparation of tumor cells

Ascites fluid was removed from a tumor-bearing animal, and centrifuged slowly (2000 **g**) for 10 minutes to sediment the cells. The cells were then resuspended in their own volume of saline. This solution was used for the incubations, within 30 minutes of withdrawal from the animal.

Preparation of tissue extracts

The same procedure was used for rats and mice, the animals providing the tissue always being of the same pure strain as those bearing the tumors.

Saline was perfused through the still beating heart to rid the animal's tissues of blood in so far as possible. The tissues to be used were removed, mixed with an equal weight of saline, homogenized for 30 seconds at 0° C. in an Ultra-turrax homogenizer (20,000 r.p.m.) and then centrifuged at 2500 **g** for 15 minutes. The supernate was used for the incubations.

In one section the lung homogenate was boiled for five minutes before centrifugation, to make the " protein-free " lung extract.

In the cell-fraction experiments the tissue was homogenized in a Potter homogenizer (1000 r.p.m.) for 5 minutes, instead of the Ultra-turrax to avoid damaging nuclei. The solution was strained to remove large particles and then centrifuged in the Spinco " 40 rotor " at 40,000 r.p.m. (150,000 **g**) for 30 minutes. Both the supernate and sediment were used in incubations, the sediment, either whole or rehomogenized for 30 seconds in the Ultra-turrax, being resuspended in saline, 1 c.c. for 0·2 g. tissue.

Incubation

The tissue extracts or controls prepared as described were then incubated with the tumor cell solutions for 2 hours at 37° with agitation, in a ratio of 3 volumes extract to one volume of ascites cell solution. At the end of the incubation the mixture was immediately injected intraperitoneally into fresh animals of the same strain, generally 5×10^6 cells per animal.

In some cases, after 2, 3, or 4 hours of incubation, smears of each tube were made and stained by the May–Gruenwald Giesma technique for microscopic examination.

In the *in vivo* experiments the extract of lung tissue, prepared as above, was injected intraperitoneally several days after the tumor cells, without incubation.

RESULTS

pH: The pH was between 6·5 and 7·0 in all incubation tubes.

Microscopic appearance

No striking cytolytic effects such as described by Druckrey for the Yoshida tumor (Steinhoff, Flaschenträger, and Bannasch, 1958) were noted with either

of the tumors used here. There was a tendency for the tumor cells incubated with lung or spleen extracts to show more nuclear pyknosis, more cell shrinkage and more cell membrane rupture than the controls. Unfortunately, however, the microscopic appearance could not always be used as a reliable guide to the viability of the cells on reinjection, in either the G–6 or the Ehrlich ascites tumor.

Results on reinjection—G–6 tumor

(1) *Whole-tissue extracts* (*Tables I, II, III*).—It was found that the extracts prepared from lung or spleen had a definite inhibitive effect on the growth of this ascites tumor ($p < 0{\cdot}01$). The extract from liver (Table III) was without effect. Extract made from the lungs of animals bearing the G–6 tumor was as effective as that made from lungs of healthy animals (Table II). The " protein-free " lung extract had no inhibitive effect (Table II).

TABLE I.—*Development of Ascites in Rats Injected Intraperitoneally with G–6 Ascites Tumour Cells Variously Treated*

Ascite cells incubated with :	Group 1 Saline	2 Whole lung extract	3 Whole spleen extract
Rat 1	385	0	0
2	528	0	0
3	300	0	0
4	396	2	0
5	432	300	0

The numbers represent the total number of ascites cells (in millions) found in each of the fifteen animals, all killed 20 days after tumor inoculation. i.e. Quantity of ascitic fluid (c.c.) times cell count (per c.c.) times 10^{-6}.

TABLE II.—*Development of Ascites in Rats Injected Intraperitoneally with G–6 Ascites Tumour Cells Variously Treated*

Ascites cells incubated with :	Group 1 Saline	2 PFLE	3 WLE	4 WSE	5 WLE-T
Rat 1	528	468	3	100	0
2	555	648	0	190	0
3	270	288	0	150	0
4	0	240	0	756	0

The numbers represent the total number of ascites cells (millions) found in each of the twenty animals, all killed 15 days after tumor inoculation. PFLE : protein-free lung extract, i.e. lung extract heated to 100° C. for five minutes before centrifugation. WLE : whole lung extract. WSE : whole spleen extract. WLE-T : whole lung extract made from lungs of rats bearing the ascites tumor.

(2) In vivo *injection*.—Injection of the whole lung extracts into rats 3 and again 5 days after ascites cell injection resulted in a slight but so far not significant inhibition in tumor growth. These intraperitoneal injections were not toxic to the animals systemically nor was there any evidence of local damage.

(3) *Cell fraction studies*.—Here incubation of G–6 cells was carried out with different ultracentrifuge fractions of rat lung tissue. The results of one such

TABLE III.—*Survival Times After Inoculation with G–6 Ascites Tumor Cells Variously Treated*

Cells incubated with :	Group 1 Saline	2 Liver E	3 SLE	4 Sed LE	5 Sed LERH
Rat 1	15	16	15	20	37
2	15	17	16	23	45+
3	16	17	17	23	45+
4	17	18	17	21*	45+
5	23	19	18	—	45+

Showing survival times in days of 24 rats, after tumor inoculation. Liver E : whole liver extract. Has no connection with ultracentrifuge studies. Groups 1 and 2 form one experiment, groups 1, 3, 4, 5 another. SLE : supernate of lung extract centrifuged at 150,000g for 30 minutes. Sed LE : sediment of lung extract. Sed LERH : sediment of lung extract rehomogenized in the ultraturrax. * : this animal lost its identification but almost certainly belongs in group 4. 45+ indicates animal was living and well after 45 days.

experiment are presented in Table III. It is seen that the supernate fraction, containing the cytoplasmic proteins (J. Chauveau, personal communication) had no inhibitive effect ; the " whole sediment " fraction, containing nuclei, mitochondria, and " microsomes " (J. Chauveau, personal communication ; De Duve *et al.*, 1955) had a slight effect (borderline significance) ; the " rehomogenized sediment " fraction, containing broken nuclei and generally undamaged mitochondria and " microsomes ", had a definite significant effect ($p < 0{\cdot}01$).

Another similar experiment tended to show similar results, but with a definite effect also in the " whole sediment " fraction.

Results on reinjection—Ehrlich tumor

From Table IV it is evident that in the Ehrlich ascites tumor likewise, lung and spleen extracts from pure-bred mice of the same strain definitely inhibited the growth of the tumor ($p < 0{\cdot}01$). Again the effect was not found in " protein-free " lung.

TABLE IV.—*Survival Times After Inoculation with Ehrlich Ascites Tumor Cells Variously Ttreated*

Ascites cells incubated with :	Group 1 Saline	2 Protein-free lung	3 Whole lung	4 Whole spleen
Rat 1	30	24	120+	18*
2	30	27	120+	45*
3	32	28	120+	120+
4	37	30	120+	120+
5	37	—	120+	120+
6	41	—	120+	120+
7	65	—	120+	120+
8	120+	—	120+	120+

Showing survival times in days of 28 mice after tumor inoculation. 120+ indicates animal was living and well after 120 days. * : indicates animal died *without* ascites. All deaths not so marked occurred with definite ascites on autopsy.

In another experiment it was found, surprisingly, that rabbit lung, prepared in the same manner, did not have the inhibitive effect found in the mouse lung.

DISCUSSION

From these results, as well as Druckrey's with the Yoshida tumor, it seems reasonable to conclude that there is a factor capable of inhibiting ascites cell growth, at least in the lung and spleen of the species studied.

It is to be noted that the rat lung tissue was effective against the G–6 tumor, although this tumor in its normal form readily metastasizes to the lung.

Since the protein-free lung extracts were always without effect, the inhibiting factor should be looked for among the proteins. Since the tissue source was always the same animal strain as that bearing the tumor, and since a number of specific immunological trials for reaction between lung tissue and tumor were always negative, we can almost certainly rule out the possibility that the inhibiting factor is an antibody.

The results of the ultracentrifuge experiments make it likely that the factor is found in cell nuclei, since the rehomogenized sediment fraction appeared much more active than the whole sediment fraction, from which it differed presumably only in having its nuclei broken and nuclear protein released. It has not yet been possible to do further subfractionation studies due to the amount of tissue needed for inhibition; thus a mitochondrial or microsomal location for the factor is still possible, but the cytoplasmic protein fraction has been ruled out in two experiments.

It is interesting that the factor is present in lung and spleen but apparently not at all in liver similarly prepared. (Druckrey found several other tissues also relatively or absolutely lacking in the factor, and claims that serum not only lacks inhibitive power but actually enhances the ascitic growth. Landschutz (1956), however, claims to have noted distinct inhibition of several ascites tumors by human serum. From these differences one might be led to suspect some cellular enzyme found in the lung and spleen but relatively little in the liver. ATPase or Hexokinase would fit these conditions in the rat (Spector, 1956) but it would be rather surprising to find the effect principally in the nuclear fraction if one of these enzymes were responsible.

Further work is needed to isolate the factor and test whether other, non-ascitic, tumors could be affected, and whether normal cells are not damaged as well. It may be possible to answer the latter question to some extent in tissue culture. So far no damage to the animal, locally or systemically, has been found, at least with the intraperitoneal injections used.

The rough *in vivo* experiments, which did show some tumor inhibition even with only two tissue injections, timed arbitrarily, may show promise.

SUMMARY AND CONCLUSIONS

The effect of a normal tissue factor, previously found to inhibit the growth of Yoshida ascites cells after *in vitro* incubation, has been investigated on several ascites tumors of the rat and mouse.

Such a factor was demonstrated in rat lung and spleen, inhibiting the growth of the G–6 ascites tumor, and in mouse lung and spleen, inhibiting the growth of the Ehrlich ascites tumor. The factor is not found in liver tissue. It is found in lung tissue from animals bearing the tumor under investigation as well as in lung tissue from normal animals. The factor is protein in nature, but is

absent from the lung cytoplasmic protein; it is present most probably in the nuclear fraction. The tissue extracts used are not toxic on intraperitoneal injection and have a slight ascites-inhibiting effect *in vivo*.

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SERIAL IRRADIATION OF MOUSE TUMOURS: EFFECTS ON TUMOUR ESTABLISHMENT TIME AND GROWTH RATE

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Received for publication October 19, 1959

SOME authors have shown that a reduced growth rate occurs in animal tumours after single or repeated irradiations. Russ (1924) observed this in post-irradiation tumours to the extent of one-quarter of the control growth rates. Sugiura (1934) also observed this effect in recurring Sarcoma 180 tumours after irradiation with a sub-lethal dose. Snellman (1935) reported similar findings in the Jensen rat sarcoma subjected to serial irradiations, even after transplantation into fresh animals. However, Hill, Morton and Witherbee (1919) found no changes in tumour establishment time or growth rate after serially irradiating Adenocarcinoma 63 for 14 generations.

The present communication reports the effects of serial irradiations on the establishment times and growth rates of Sarcoma 37 and two homologous transplantable mouse tumours.

MATERIALS AND METHODS

RIII strain inbred mice were used throughout this study. The transplantable tumours, from which the serially irradiated lines were developed, were Sarcoma 37 and two homologous tumours of the RIII strain—a spindle-celled sarcoma (BP1) and a mammary adenocarcinoma (MV212). The establishment of the irradiated lines has been described in a previous communication (Pearson, 1959). Three irradiated lines were treated with sub-lethal doses (Sarcoma 37 " B " line, BP1 " F " line and MV212 " G " line) and the fourth treated with half-lethal doses at each stage (Sarcoma 37 " D " line).

Recurring post-irradiation tumours or those from untreated sub-line passages were used for donor material in the growth rate estimations. 1 mm.^{3} portions from the cortex of parent tumours were inoculated subcutaneously into the right flanks of the experimental animals. Tumour area was employed in the comparisons and was calculated as the product of the major and minor axes measured by calipers.

The growth rates of Sarcoma 37 " B " line tumours were measured at the 12th irradiation stage after 5, 15, and 32 untreated sub-line passages (B12/5, B12/15 and B12/32) and at the 17th stage after 3 sub-line passages (B17/3). Sarcoma 37 " D " line tumours were measured at the 10th irradiation stage after 1 and 8 sub-line passages (D10/1 and D10/8). The growth rates of the " F " and " G " irradiated lines of the homologous spindle-celled sarcoma and mammary adenocarcinoma, were made at the 1st sub-line passage from the 7th

and 9th irradiation stages respectively. The numbers of mice employed in each group are shown in brackets in Fig. 1–4.

RESULTS

The average growth rates of tumour groups from the control and irradiated " B " lines of Sarcoma 37 are shown in Fig. 1. The marked increase in tumour establishment time and reduction in growth rate was of the same order for the 5th, 15th and 32nd sub-line passages from the 12th irradiation stage. Tumours from the 17th irradiation stage exhibited a further increase in the establishment time. Tumours from the 10th irradiation stage of the Sarcoma 37 " D " line

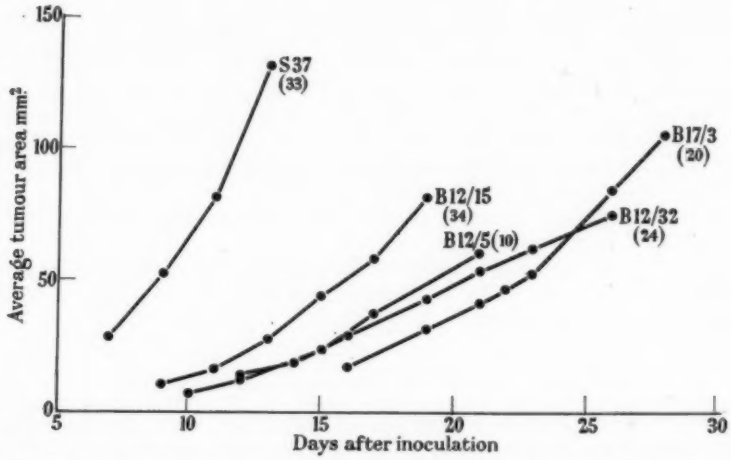

FIG. 1.—Growth rates of Sarcoma 37 and its serially irradiated " B " line. Figures in parentheses denote numbers of mice employed in each group.

showed similar but less marked growth changes (Fig. 2) and again sub-line passages without further irradiation did not alter this effect.

The increase in establishment time occurred gradually. This period (calculated from inoculation to the attainment of about 15 mm.² in area) was 7 days up to the 4th irradiation stage in the " B " line, 8–13 days from the 5th to 8th stages and 11–16 days for the remaining stages. " D " line tumours exhibited an establishment time of 7–8 days up to the 3rd stage and thereafter varied from 11–16 days.

The growth rates of the homologous tumours BP1 and MV212 and their respective irradiated lines are shown in Fig. 3 and 4. No changes in tumour establishment times or growth rates were observed.

DISCUSSION

The growth rate effects of serial irradiations on Sarcoma 37 were found to be the result of an intrinsic alteration in cell behaviour, as the observed changes were reproduced after sub-line passages without further irradiations. These

findings are therefore comparable with those of Snellman (1935) on the Jensen rat sarcoma. The increase in establishment time and decrease in growth rate were observed in both irradiated lines of Sarcoma 37, but no changes of this nature were observed in the two irradiated lines of the homologous tumours.

A constant and permanent change in growth rate in all the irradiated tumours has therefore not been demonstrated in this study ; however, it has been shown that a permanent change is possible. This fact should therefore be taken into account where the assessment of radiosensitivity is based on tumour growth rate after a single irradiation dose, as employed by Dittrich, Hohne and Schubert (1956), particularly where non-strain-specific tumours are employed.

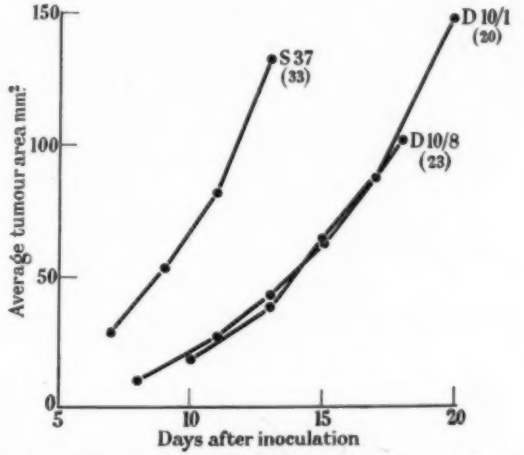

FIG. 2.—Growth rates of Sarcoma 37 and its serially irradiated "D" line. Figures in parentheses denote numbers of mice employed in each group.

SUMMARY

1. Growth rate comparisons have been made on tumour transplants from untreated and serially irradiated lines of Sarcoma 37 and two homologous mouse tumours.

2. Sarcoma 37 serially irradiated with sub-lethal doses showed an increase in tumour establishment time and a decrease in growth rate. A similar but less marked effect was demonstrated when this tumour was serially irradiated with half-lethal doses at each stage.

3. These growth changes were not altered by sub-line passages without further irradiation.

4. Serially irradiated lines of two homologous tumours showed no variations in establishment time or growth rate when compared with their respective controls.

My thanks are due to Mr. F. W. Butcher and Miss G. V. Adam for assistance with the animal experiments.

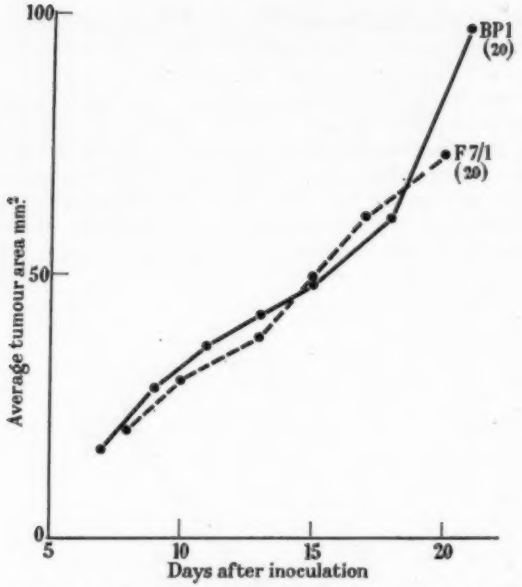

FIG. 3.—Growth rates of spindle-celled sarcoma BP1 and its serially irradiated "F" line. Figures in parentheses denote numbers of mice employed in each group.

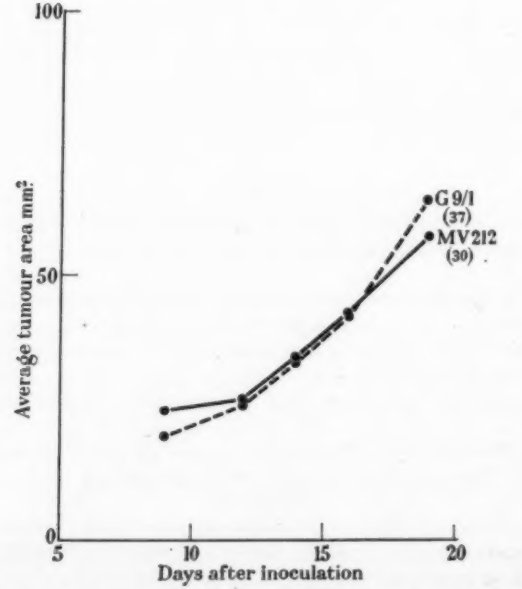

FIG. 4.—Growth rates of mammary adenocarcinoma MV212 and its serially irradiated "G" line. Figures in parentheses denote numbers of mice employed in each group.

The expenses of this research were defrayed from a block grant by the British Empire Cancer Campaign.

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THE EFFECT OF SARCOMA 37 ON THE INTRACELLULAR DISTRIBUTION OF MOUSE LIVER CATALASE

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Received for publication October 23, 1959

MUCH work has been published on the effect on liver catalase of tumour growth, and of the injection of whole or semi-purified tumour homogenates, but in all these studies the sole criterion of activity has been a simple depression in catalase level. An increasingly large number of substances apparently unrelated to tumour tissue, have been shown to depress liver catalase activity on injection, e.g. methyl *bis* (B-chlorethyl) amine, 9 : 10-dimethyl-1 : 2-benzanthracene, butter yellow and its 3'methyl demative, 4'-amino-2 : 3-dimethylaminoazobenzene, B-naphthylamine, benzpyrene (Adams and Roe, 1953), homogenised normal spleen tissue (Day, Gabrielson and Lipkind, 1954), aminotriazole (Heim, Appleman and Pyrform, 1955) allyl isopropyl acetyl carbamide (Schmidt, Figen and Schwarz, 1955), cysteine and a number of its derivatives (Hirai and Deutsch, 1958), thiourea (Margoliash, 1958, personal communication), testosterone and 17-methyl testosterone (Adams, 1960).

There is little direct evidence about the mechanism by which these substances depress catalase activity *in vivo*. Probably none of them directly inhibit the enzyme. Amino triazole acts *in vitro* by combining with catalase-hydrogen peroxide Complex I (Margoliash and Norogrodsky, 1958). However this substance is characterised *in vivo* by an extremely rapid action—liver catalase activity being reduced almost to zero in a few hours. It is well known that hydrogen peroxide destroys catalase and therefore one possible explanation is that these substances affect catalase activity through the production of hydrogen peroxide during their metabolism. Certainly if every substance which gives rise to H_2O_2 *in vivo* depresses catalase activity, such depressions are completely non-specific. Has then tumour growth or tumour tissue any specific action on liver catalase ?

Adams and Burgess (1959*a*) recently showed that when liver slices were incubated *in vitro* in a phosphate saline medium, catalase migrated from the large granules into the extra particulate cytoplasm (EPC). This observation led to the suggestion (Adams, 1960) that such catalase migration may occur *in vivo* and that catalase is synthesised only in the large granules. On this view the EPC level would be maintained by catalase derived from the granules by migration through the granule membranes. If this is correct, then the permeability of the granule membranes will be one of the factors determining the EPC catalase level. Adams (1960) also suggested that, in the simplest analysis, the granule/EPC catalase distribution ratio would provide an approximate index of granule permeability. He showed that after the injection of cysteine, methyl butter yellow, or thiourea, depressions in catalase activity were observed in both granule and EPC

fractions, but that these changes occurred without alteration in the granule/EPC distribution ratio. Further evidence in the same paper showed that androgenic hormones altered the granule/EPC distribution ratio in a way consistent with the supposition that they act by increasing the permeability of the large-granule membranes to catalase. Adams (1951) found that there appeared to be an antagonism between the action of testicular and adrenal hormones, and that of tumour homogenate or catalase activity.

The purpose of this paper is to make a preliminary study of the action of tumour growth and tumour homogenate on intracellular catalase distribution.

MATERIALS AND METHODS

Animals.—Young adult mice of the 101 and CBA strains and of an albino strain derived from AKR were used. These animals were all bred in this laboratory by brother-sister mating, but some AKR-substrain mice were obtained direct from the Laboratory Animal Bureau.

Preparation of liver fractions and estimation of liver catalase activity.—Catalase estimations were made on whole liver homogenates, and on granule and EPC fractions. The method of estimation of catalase activity has been fully described in previous publications (Adams, 1950, 1952) and the preparation of the homogenates and fractions by Adams and Burgess (1957, 1959*a*). Ethanol (final concentration 0·01 M.) was added to all catalase containing solutions to prevent loss of catalase due to " Complex II " formation (Chance, 1950 ; Adams and Burgess, 1959*b*).

Tumour.—Sarcoma 37 was obtained originally from the Imperial Cancer Research Fund Laboratories and maintained by serial passage in this laboratory.

Triton × 100.—This non-ionic detergent (kindly given to us by Charles Lennig & Co.) was used at a final concentration of 0·25 per cent v/v to disrupt large granules and liberate their catalase activity into solution.

RESULTS

The injection of normal tissue

Adams (1950), working with homogenates containing principally EPC fraction, found that the injection of 50 mg. doses of normal tissue did not depress catalase activity significantly. In view of the finding by Day *et al.* (1954) that injections of homogenised spleen did depress liver catalase activity, the effect of larger doses of normal tissue on total, granule, and EPC catalase was investigated. The normal tissue used was a mixture of the liver, spleen, and kidneys taken from sufficient CBA mice to provide the total required. The mixed tissues were homogenised (50 strokes with a Ten Broeck grinder) and injected subcutaneously in 100 and 200 mg. doses into AKR-substrain female mice. As shown in Fig. 1 the lower dose produced only a slight depression in catalase activity, but the higher dose resulted in considerable depression in catalase activity in both granule and EPC fractions. However, the granule/EPC catalase distribution ratio remained almost unaltered through the course of the experiment. Fig. 1 also shows that the effect of a single injection of homogenised rat liver (100 mg.) was similar to that produced by the same dose of mouse tissue. Closely similar results, i.e. depression in catalase activity with little or no alteration in the distribution ratio,

were obtained after the injection of cysteine, thiourea, and butter yellow (Adams, 1960).

The injection of tumour tissue

Fig. 2 shows the result of the injection of 100 mg. and 200 mg. of homogenate prepared in a similar way from Sarcoma 37 into AKR-substrain females. As with normal tissue, depressions in catalase activity were observed, but with the

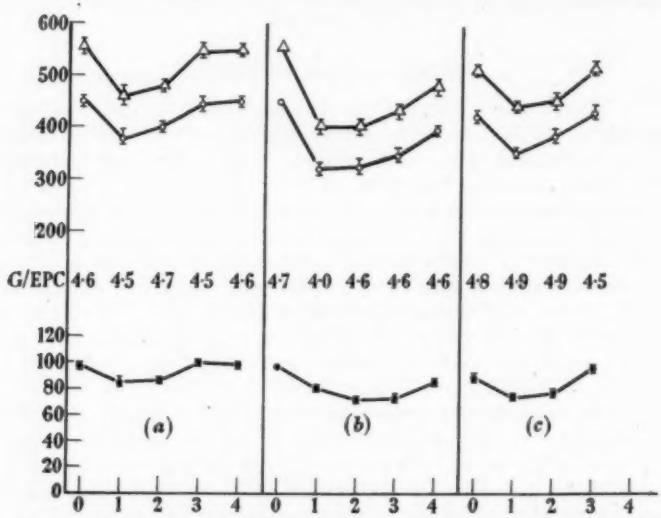

FIG. 1.—The effect of the injection of homogenised normal tissue on the catalase activity and granule/EPC catalase distribution ratio of AKR-substrain ♀ mice. (*a*) and (*b*) 100 mg. and 200 mg. of mixed liver kidney and spleen from CBA mice. (*c*) 100 mg. of similar tissue from albino rats. Injection at 0 days.

△——△ Total catalase. ○——○ Granule catalase.
●——● EPC catalase.

Results are expressed in this and subsequent figures as arithmetic means ± Std. errors of means. Twelve control and eight treated animals/group.
Ordinate.—Catalase activity in arbitrary units/mg. N.
Abscissae.—Time in days.

difference that the granule/EPC catalase distribution ratio increased considerably. Fig. 3 shows the results of the injection of 100 mg. of normal tissue, and 100 mg. of tumour tissue, into CBA males. The normal tissue resulted in depression in catalase activity without alteration in the granule/EPC distribution ratio (3·3 in normal males). The injection of tumour homogenate resulted in an increase in the ratio to 4·6.

Tumour growth

The effect of tumour growth on the catalase activity and granule/EPC distribution ratio in AKR-substrain female and 101 male and female mice is shown

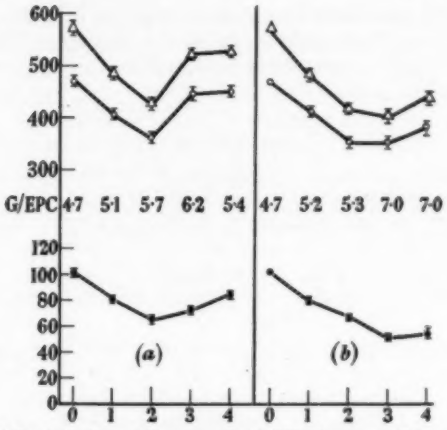

FIG. 2.—The effect of the injection of homogenised S37 tissue on the catalase activity and granule/EPC catalase distribution ratio of AKR-substrain ♀ mice. (*a*) 100 mg. (*b*) 200 mg. injected at 0 days.

△——△ Total catalase. ○——○ Granule catalase.
●——● EPC catalase.

Ordinate.—Catalase activity in arbitrary units/mg. N.
Abscissae.—Time in days.

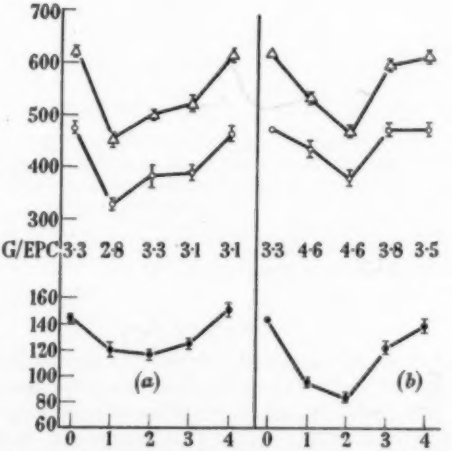

FIG. 3.—The effect of the injection of homogenised tissue on the catalase activity and granule/EPC catalase distribution ratio of CBA ♂ mice.
(*a*) 100 mg. liver-kidney-spleen taken from AKR mice.
(*b*) 100 mg. S37 tissue.

△——△ Total catalase. ○——○ Granule catalase.
●——● EPC catalase.

Ordinate.—Catalase activity in arbitrary units/mg. N.
Abscissae.—Time in days.

in Fig. 4. Progressive decreases in catalase activity in both granules and EPC were associated with progressive increases in the granule/EPC distribution ratio. In the AKR-substrain mice considerable tumour necrosis was found, but little or none in the 101 mice.

DISCUSSION

Surprisingly enough perhaps comparatively little work has been done on direct comparison of the effect of the injection of normal and tumour tissue on liver catalase. Adams (1950) (measuring principally EPC catalase) found that

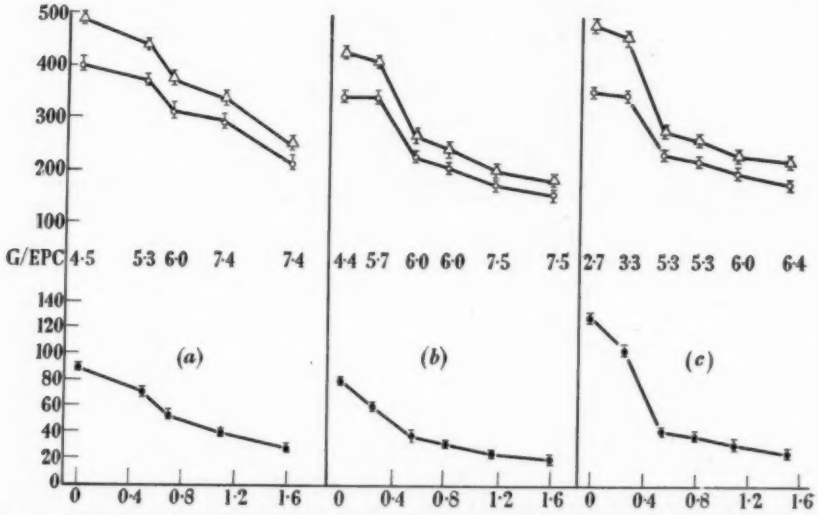

FIG. 4.—Effect of S37 tumour growth on catalase activity and granule/EPC catalase distribution ratio of (a) AKR-substrain ♀ mice. (b) 101 ♀ mice. (c) 101 ♂ mice. The tumour bearing mice were formed into groups of 6–8 animals bearing tumours of similar size and the tumour weights averaged to give the points on the graphs.

△——△ Total catalase. ○——○ Granule catalase.
●——● EPC catalase.

Ordinate.—Catalase activity in arbitrary units/mg. N.
Abscissae.—Tumour weight in grams.

the injection of 50 mg. dose of normal tissue did not depress mouse liver catalase significantly, but 50 mg. dose of S37 did. The present work shows clearly that catalase depressions may easily be obtained after the injection of normal tissue, provided that the dose is sufficiently high. This depression of catalase activity by normal tissue injection confirms the result of Day *et al.* (1954). In fact if only *total* catalase activity is considered normal tissue homogenate may depress catalase to the same extent, dose for dose, as tumour homogenate. The effect on the intracellular (granule/EPC) catalase distribution ratio was however quite different. Normal tissue homogenate did not alter this ratio in either sex, and this resembled thiourea, cysteine and methyl butter yellow in this respect (Adams,

1960). On the other hand, tumour homogenate increased the granule/EPC ratio considerably in both sexes, and the same phenomenon was seen during tumour growth. The results suggest that injected tumour tissue has two separate actions on catalase. Firstly, the total enzyme level is decreased, an effect which also occurs with normal tissue, and secondly, superimposed on this, there is an increase in the granule/EPC distribution ratio. This action on the distribution ratio, which is apparently specific, shows itself principally by a decrease in the EPC level. Thus if a comparison is made of the action of tumour homogenate and normal tissue homogenate on the EPC catalase level only, tumour tissue will produce a greater depression compared with normal tissue. This accounts for the results previously reported (Adams, 1950) that 50 mg. tumour homogenate significantly depressed the (EPC) catalase activity whereas no depression was caused by normal tissue homogenate.

The results given here, taken in conjunction with those summarised in the introduction, make it clear that a simple catalase depression is not in the least a specific action of tumour tissue or of tumour growth.

It seems also quite clear that the fractions derived from tumour tissue by various investigators cannot, on the available evidence, be said to contain any specific tumour agent. These fractions have been tested solely on the basis of their ability to depress catalase activity *in vivo* after injection. There are obviously many substances which could be present in varying amounts in tumour tissue and in normal tissue, which will produce a catalase depression when injected in adequate dosage.

Tumour growth resulted in a progressive fall in catalase activity, and a progressive increase in granule/EPC distribution ratio. However the catalase activities in these mice fell to lower levels than those observed after injections of tissue homogenates. This point will be dealt with in a future publication.

Adams (1960) showed that androgenic hormones injected into female mice exerted a specific effect in decreasing the liver catalase granule/EPC distribution ratio from the normal value of about 5·0, to the normal male value of about 3–3. This was interpreted as showing that the hormones increase the permeability of the granule membranes to catalase. On the same view, the increase in distribution ratio caused by tumour growth and tumour tissue would result from a decrease in the permeability of the large-granule membranes to catalase. The antagonistic action of tumour tissue and hormones reported by Adams (1951) appears therefore to result from their opposite effects on granule membrane permeability.

SUMMARY

(1) Depression of liver catalase activity results equally well from injections of S37 homogenate or normal tissue homogenates.

(2) S37 homogenate increases the granule/EPC catalase distribution ratio, but normal tissue does not.

(3) It is concluded that S37 homogenate contains an agent which decreases the permeability of the granule membranes to catalase.

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THE EFFECTS OF SOME POLYCYCLIC HYDROCARBONS ON MOUSE LIVER –SH LEVELS

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Received for publication October 29, 1959

THE involvement of sulphydryl groups in the biological activity of carcinogenic agents is a subject which has frequently been discussed. Unfortunately, very few attempts have been made to measure any actual changes in tissue –SH levels induced by carcinogens.

Estimations of the glutathione level of mouse skin after treatment with various hydrocarbons were made by Crabtree (1946). The non-carcinogens, naphthalene, anthracene and phenanthrene caused distinct falls as compared with normal levels but dibenzanthracene and 3 : 4 benzpyrene had no effect. More recently DiPaolo and Niedbala (1957) could find no changes induced by anthracene in the –SH levels of dermis and epidermis of mouse skin. On the other hand they found 1 : 2 : 5 : 6 dibenzanthracene and 9 : 10 dimethylbenzanthracene to cause an initial rise, then a sharp fall followed by a further rise in level. This last rise persisted for as long as five days after treatment with the hydrocarbon.

Estimations of water extractable –SH from normal and benzpyrene treated rat livers were made by Rondoni and Boretti (1947). It was concluded that, at intervals between 24 hours and 21 days after treatment there was a fall in liver –SH in the treated animals. Later, however, Rondoni (1955) himself suggested that as the measured variations were very small the conclusions should be treated with reserve.

Based on the argument that serum or plasma –SH levels should be a reflection of tissue levels Wood and Kraynak (1953) determined the plasma –SH level of dogs at intervals after intravenous injections of anthracene or 3 : 4 benzpyrene and the serum –SH levels of rabbits after similar treatments. Between ½ hour and 6 hours after treatment benzpyrene caused significant falls in –SH content but anthracene had no effect on the normal level.

The present work records the results obtained from measurements of the liver –SH levels of mice at relatively short intervals after the intravenous injection of polycyclic hydrocarbons. Liver was selected for these studies as being an organ where hydrocarbons are known to be deposited and metabolised. It was thought that any variations found might help to throw light on the problems associated with questions of the metabolism of hydrocarbons.

EXPERIMENTAL

All mice used in these experiments have been taken from a stock of inbred Strong A animals maintained in this laboratory. Each batch of mice used has

comprised animals born within 1–2 days of one another. The actual ages of the batches are recorded with the results.

The hydrocarbons used were anthracene, pyrene, perylene, 1 : 2 : 5 : 6 dibenzanthracene and 3 : 4 benzpyrene. The hydrocarbons were prepared as colloids in distilled water. Each experimental animal received a single intravenous injection via the tail vein of 0·5 mg. of hydrocarbon in 0·5 ml. of distilled water.

Liver –SH levels were determined by the method already described by Calcutt and Doxey (1959). This involves the immersion of a known weight of liver slices (weights of 90–160 mg. were used) in a measured volume of a standard solution of *p*-chloromercuribenzoic acid (C.M.B.) and after an appropriate time interval for the reaction to take place the potentiometric titration of any unchanged C.M.B. with a standard solution of cysteine hydrochloride. During the present work every titration has been run in duplicate, the two runs being done at the same time on completely separate sets of instruments. With certain exceptions during a run with dibenzanthracene (this will be referred to later) end points on the two sets of equipment were either completely in agreement or within 0·1 ml. of one another. For calculation purposes the mean of the two results was used.

RESULTS

The results are given under the headings of the individual hydrocarbons. For each batch of mice a mean figure and standard deviation for untreated animals has been determined. In each case 10 animals have been used for this purpose.

Anthracene.—Female mice aged 25 weeks. Control value 30·4 $\pm$ 2·8 μg. of –SH per 100 mg. wet weight of liver. Results are shown in Fig. 1. Apart from a very sharp rise in –SH level occurring $\frac{1}{2}$–1 hour after injection of the hydrocarbon, levels tend to fall consistently below the control value; this still being apparent 24 hours after treatment.

Pyrene.—Male mice aged 26 weeks. Control value 32·9 $\pm$ 3·5 μg. of –SH per 100 mg. wet weight of liver. Results are shown in Fig. 2. After an initial rise in level similar to that found with anthracene the level falls below the control value for a few hours and then returns to the normal range.

Perylene.—Female mice aged 25 weeks Control value 29·7 $\pm$ 4·6 μg. of –SH per 100 mg. wet weight of liver. Results are shown in Fig. 3. Again there was an early rise ($\frac{1}{2}$ hour) in –SH level, but this time apparently succeeded by a further rise at about 2 hours after treatment. This was followed by a return to the normal or a possibly slightly enhanced level. At no time up to 24 hours after injection was there any decline in –SH level below the range of normal values.

3 : 4 *Benzpyrene.*—Female mice of the same batch as the previous experiment. The results are shown in Fig. 4. As in the earlier experiments there was an initial rise in –SH value, but this time occurring at 1$\frac{1}{2}$–2 hours after treatment. This was followed by a fall to levels below the normal range and then a return to normal after about 12 hours.

1 : 2 : 5 : 6 *Dibenzanthracene.*—Male mice aged 10 weeks. Control value 32·1 $\pm$ 3·6 μg. of –SH per 100 mg. wet weight of liver. The results are shown in Fig. 5. Initially the picture closely resembled that obtained with perylene; there being an early rise succeeded by another rise at about 3$\frac{1}{2}$–4 hours. This was succeeded by a fall to levels below normal and then a gradual return to a normal level by 24 hours. At 48, 72 and 96 hours after treatment, however, the

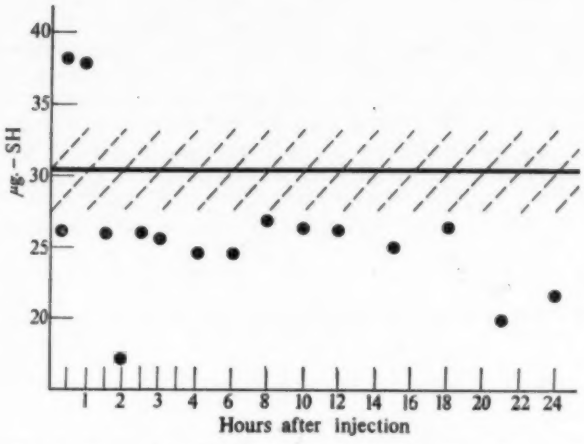

FIG. 1.—Mouse liver -SH levels after treatment with anthracene.

In this and all successive figures the mean control figure for untreated animals is shown by a heavy horizontal line; the standard deviation of the control figure is indicated by hatching and experimental points are shown as circles.

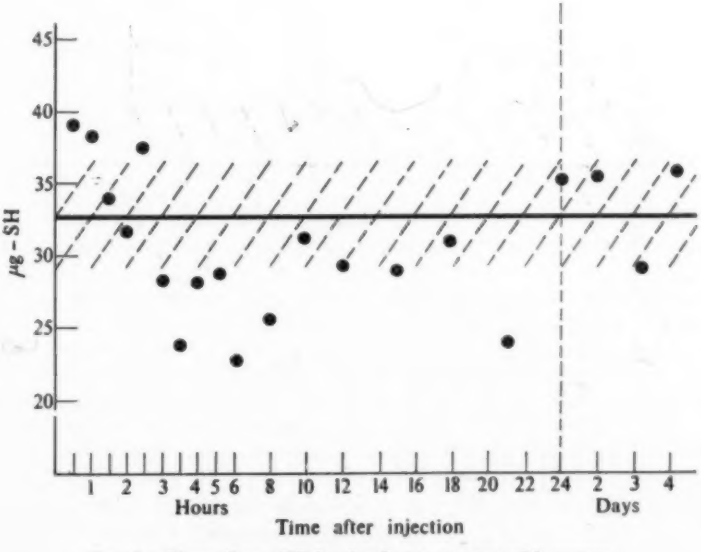

FIG. 2.—Mouse liver -SH levels after treatment with pyrene.

titration curves were unusual in that two sharp falls were recorded instead of the normal one associated with the end point. Calculated from the end points determined from the first falls in the curves enhanced –SH levels were found as

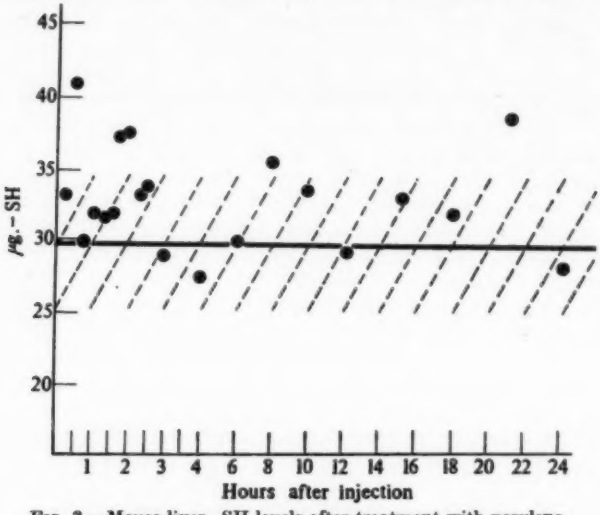

FIG. 3.—Mouse liver –SH levels after treatment with perylene.

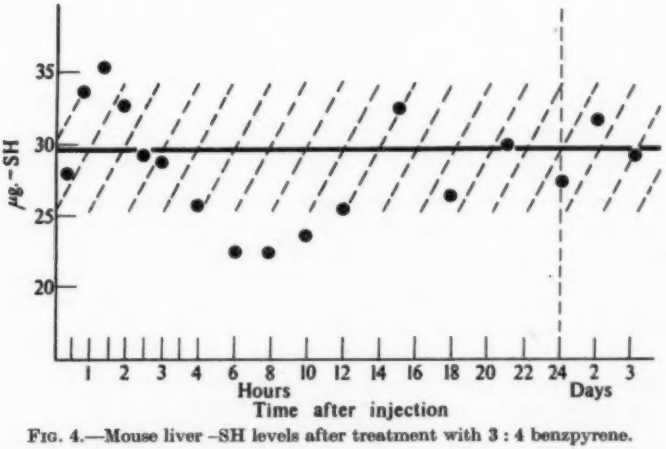

FIG. 4.—Mouse liver –SH levels after treatment with 3 : 4 benzpyrene.

shown in the figure. Calculations based upon the second end point, however, gave levels which fell strictly within the normal range.

The other finding from this data is the close correspondence between the normal control levels in the different batches of mice. The male animals show a slightly higher level than the females.

DISCUSSION

The hydrocarbons used in the present experiments fall into one or other of two groups. Anthracene, pyrene and perylene are apparently biologically inactive. Benzpyrene and dibenzanthracene, apart from being potent carcinogens, are also very active photosensitizing agents, effective in eliciting neural inductions and capable of causing mitotic abnormalities. Apart from Ilfeld's (1936) claim to have induced a hepatoma by the insertion of a pellet of dibenzanthracene into mouse liver there is no evidence suggesting any effect of any of the compounds used on liver tissue. It is against this background of the known

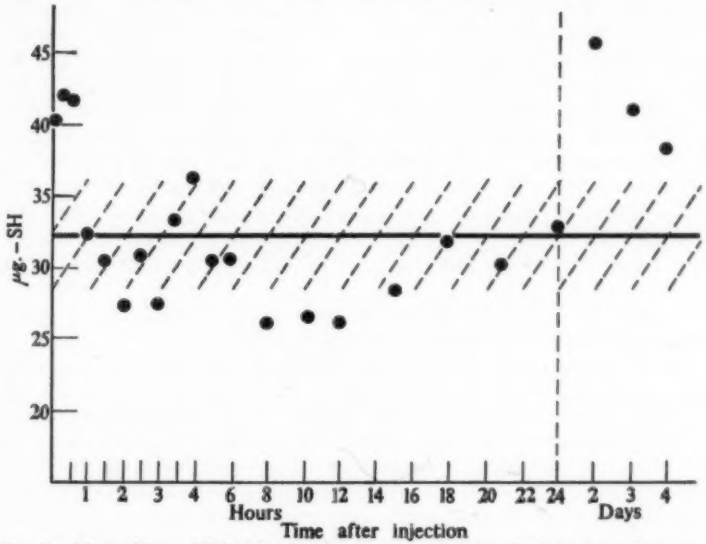

FIG. 5.—Mouse liver –SH levels after treatment with 1 : 2 : 5 : 6 dibenzanthracene.

biological activity and the known data in respect of metabolism that the present results must be considered.

The one feature displayed by all the agents tested is the early induction of a rise in liver –SH level. This parallels the finding of DiPaolo and Niedbala (1957) that dibenzanthracene and 9 : 10 dimethylbenzanthracene cause an early rise in skin –SH levels. Since these last authors applied their hydrocarbon as solutions in acetone whilst we have used aqueous colloids this rise would not appear to be due to the vehicle of introduction but to an effect of the hydrocarbon. Further, the appearance of this rise within 15 minutes in the case of dibenzanthracene but only after 1–1½ hours in the case of benzpyrene is also consistent with this effect being due to the hydrocarbon, since the introduction of the agent and handling of the animals has been identical in the two series.

After the initial rise the picture varies among the different compounds used. Both pyrene and benzpyrene show a fall to levels below the normal range. Dibenzanthracene shows a fall, then a rise and a further fall. Perylene drops to

a level which may be slightly above the normal whilst anthracene settles to a level rather below the normal figure. There is, obviously, no relationship between these behaviours and the carcinogenic or other biologic activity of the compounds concerned.

With regard to metabolism, both pyrene and benzpyrene are known to be converted to phenolic derivatives in mouse liver (Harper, 1958*a*, 1958*b*). In the case of dibenzanthracene Dobriner, Rhoads and Lavin (1939) isolated a phenolic derivative from mice and this was shown by Cason and Fieser (1940) to be 4′–8′ dihydroxydibenzanthracene. Breakdown products of this derivative were also found by Heidelberger and Wiest (1951). In the case of perylene nothing is known with regard to metabolism. Anthracene metabolism has been extensively investigated in rats and rabbits but not in mice. However, Calcutt (1959, unpublished data) isolated a derivative, apparently identical with the 1 : 2-dihydroxy-1 : 2-dihydro anthracene formed by rats, from both the urine and livers of mice treated with anthracene. It is known that anthracene in rats is partially excreted as a mercapturate (Boyland and Levi, 1936). If a similar mercapturate formation occurs in mice then this may account for the persistent low level obtained for –SH values after treatment with this hydrocarbon.

At the moment no positive conclusions can be drawn as to any relationship between fluctuations in liver –SH levels and metabolism. Further data is required in regard to the intracellular sites of –SH changes and sites of metabolic activity.

The anomalous findings in respect of dibenzanthracene at 48, 72 and 96 hours after treatment are, at the moment, unexplained.

The results obtained in the present series are not strictly comparable with previously published work since different tissues and time intervals have been used. It may, however, be noted that the picture of an initial rise followed by a fall and then a further rise is very similar to that achieved in skin with dibenzanthracene and dimethylbenzanthracene by DiPaolo and Niedbala (1957). On the basis of the presently available evidence there appears to be no correlation between carcinogenicity of the applied agent and resulting disturbances of sulphydryl level, but there may be an association with metabolic behaviour.

SUMMARY

The sulphydryl levels in the livers of mice which had received intravenous injections of 0·5 mg. of a polycyclic hydrocarbon have been determined. Anthracene, pyrene, perylene, 3 : 4 benzpyrene and 1 : 2 : 5 : 6 dibenzanthracene were used.

All five hydrocarbons caused an initial rise in liver –SH level. This occurred at various times between 15 minutes and 1½ hours after treatment. Subsequent to this rise there were fluctuations in –SH level; these varying with the hydrocarbon used.

The results are discussed in relation to the known biologic activities and metabolism of the hydrocarbons.

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THE INTERMEDIARY METABOLISM OF POLYCYCLIC HYDROCARBONS

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Received for publication October 31, 1959

PREVIOUS studies of the metabolism of pyrene and 3 : 4-benzpyrene have been reported (Harper, 1957*a*, 1958*a*, 1958*b*, 1958*c*). This work confirmed the earlier finding of Weigert and Mottram (1946) that, following injection of 3 : 4-benzpyrene into mice, two metabolic fractions, designated as X_1 and X_2, are excreted in the bile and undergo conversion to phenolic derivatives during passage through the intestine. These two fractions were identified respectively as sulphuric acid (X_1) and glucuronic acid (X_2) esters of fully aromatic benzpyrenols (Harper, 1958*b*, 1958*c*) and a similar sequence of conjugation and hydrolysis was established for the 3-pyrenol metabolite of pyrene (Harper, 1958*a*, 1958*c*). Also associated with the glucuronide fractions yielded by both pyrene and 3 : 4-benzpyrene was an acid-decomposable precursor of the parent hydrocarbon.

This work has now been extended to a range of hydrocarbons, namely 1 : 2-benzanthracene, chrysene, 20-methylcholanthrene, 1 : 2 : 5 : 6-dibenzanthracene and anthracene, utilising the same extraction and analytical procedures. Full details of these are to be found in the above publications.

MATERIALS AND METHODS

20-Methylcholanthrene (Hoffmann-La-Roche and Co. Ltd.) and anthracene (B.D.H. Ltd.) were used as purchased; the other hydrocarbons were purified by chromatography on alumina from benzene and cyclohexane followed by fractional crystallisation from ethanol, aq. ethanol or benzene. Colloidal solutions of the hydrocarbons were prepared by the method of Boyland (1932). In the case of 1 : 2 : 5 : 6-dibenzanthracene and chrysene however it was found necessary to heat both acetone solution and water to 50° C. prior to mixing.

Strong A mice, in batches of about twenty, were injected intravenously with 0·5 mg. of colloidal hydrocarbon and the distribution of metabolites within the internal organs of the body was then investigated using the same general methods of extraction and chromatographic separation previously reported for pyrene and 3 : 4 benzpyrene. A modification adopted in the case of faecal metabolites however was as follows.

The freshly voided faeces were extracted into xylene via acetone (Harper, 1957*a*) and the xylene passed through a column of silica gel (100/200 mesh) for the removal of acidic metabolites and much of the colouring matter. The filtrate from the column was evaporated under reduced pressure, the residue extracted repeatedly with boiling cyclohexane and the cyclohexane chromatographed on

silica gel. Any phenolic metabolite of the hydrocarbon was then retained as a blue fluorescent zone on the column from which it was readily eluted either with ethanol or with cyclohexane/benzene mixtures. This procedure was preferred to chromatography on alumina as used in previous studies owing to the difficulty experienced in removing small amounts of phenolic derivatives from this adsorbent.

4′-Hydroxy-1 : 2-benzanthracene (4′-benzanthrol) was synthesised by the method of Sempronj (1939). 4′-Methoxy-1 : 2-benzanthracene was prepared from this by methylation using dimethyl sulphate and excess sodium hydroxide, the reaction being continued until the bright golden yellow fluorescence of the solution had disappeared.

1-Anthrol was prepared by reduction of 9 : 10-anthraquinone-1-sulphonic acid followed by alkali fusion.

The reference conjugates, 4′-benzanthryl glucuronide and sulphate and 1-anthryl glucuronide and sulphate, were isolated from the bile and small intestines of mice injected with the parent phenols (cf. Harper, 1958*c*).

RESULTS

Three hydrocarbons, 1 : 2-benzanthracene, chrysene and 20-methylcholanthrene, yielded well defined blue fluorescent X_1 and X_2 type metabolites. Excretion was mainly via the bile and hydrolysis to phenolic derivatives and, in the case of 20-methylcholanthrene, also to an acidic derivative, occurred in the caecum and large intestine. Blue fluorescent X_1 and X_2 type metabolites were also isolated from the bile and small intestine after injection with dibenzanthracene but the yields of these were greatly reduced by comparison with the other hydrocarbons. In marked contrast to this behaviour, however, was the failure of anthracene to yield any material that was recognisable as a metabolite under the same conditions of extraction and analysis.

The X_1 type metabolites were readily hydrolysed by cold dilute mineral acid and by takadiastase. β-Glucuronidase was without effect. The X_2 type metabolites on the other hand, were unaffected by treatment with mineral acid in the cold but hydrolysis occurred on prolonged heating under nitrogen with strong acid (6N HCl). Rapid hydrolysis also occurred on incubation with β-glucuronidase but this effect was nullified in the presence of 10^{-2} M boiled saccharate solution.

In view of these properties, and by analogy with the behaviour of the conjugates of 3-pyrenol and benzpyrenols (Harper, 1958*c*), the two metabolic fractions were concluded to be sulphuric and glucuronic acid esters respectively. The essential problem therefore was the identification of the hydrocarbon moieties of the conjugates.

(*A*) 1 : 2-*Benzanthracene*

The identification of the phenolic derivative of 1 : 2-benzanthracene excreted in the faeces of rats and mice was achieved by methylation and spectroscopic comparison with known methoxy-benzanthracenes (Berenblum and Schoental, 1943). On this basis the 4′-methoxy derivative was selected as being identical with the methylated metabolite although the presence of absorbing impurity limited comparison to the long wave systems (above 300 mμ) of the absorption spectra. A characteristic feature of the spectrum was a band at 391·5 mμ in

hexane but, perhaps surprisingly, the graphical data reported for the methylated metabolite does not include the sharp prominent maximum at 311·5 mμ (Fig. 2).

In the present work attempts have been made to confirm this identification by comparison of the conjugated and free phenolic metabolite with the synthetic analogues derived from 4′-benzanthrol.

(a) *The conjugated metabolites*

The physical and chemical properties of the X_1 and X_2 type metabolites were respectively identical with those of 4′-benzanthryl sulphate and glucuronide.

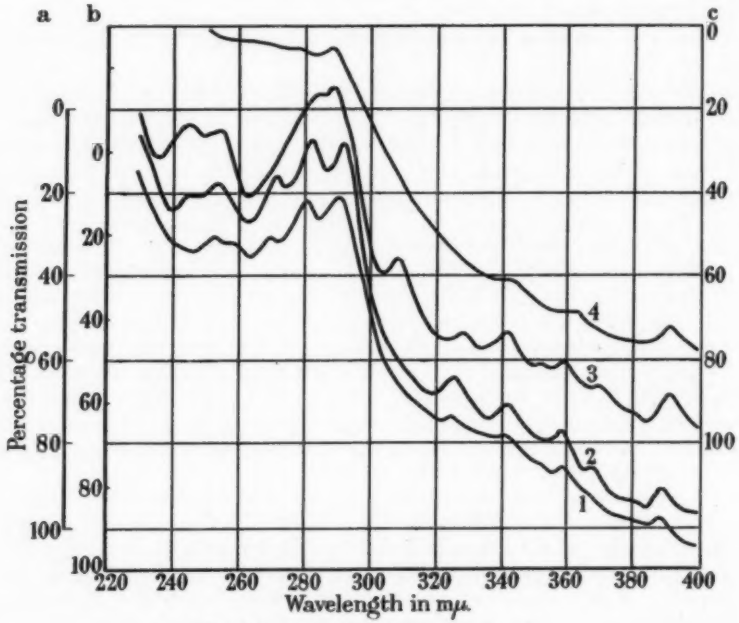


Fig. 1.—Absorption spectra in ethanol.

1. X_1 type metabolite of benzanthracene (*b* ordinates).
2. 4′-benzanthryl sulphate (*a* ordinates).
3. 4′-benzanthryl glucuronide (*c* ordinates).
4. X_2 type metabolite of benzanthracene (*c* ordinates).

The one exception was observed with the X_2 type metabolite for, on acid hydrolysis, a small amount of free 1 : 2-benzanthracene was liberated in addition to a phenolic derivative. The presence of an acid-decomposable precursor of the hydrocarbon was therefore indicated.

The absorption spectra of the two sets of conjugates are recorded in Fig. 1. The resemblance between the two types of spectra is at once apparent and provides confirmatory evidence of the conjugated nature of the hydrocarbon metabolites. In Table I however are recorded the detailed positions of the absorption bands and reference to this data reveals that slight differences exist

in the location of certain bands.* (Failure to record a band at or near 308 mμ in the spectrum of the X_2 type metabolite is possibly due to the presence of absorbing impurity. This was in fact present in one sample of the metabolite isolated from stored bile.)

A possible explanation of these differences is that the hydrocarbon metabolites consist predominantly of the conjugates of 4′-benzanthrol but that smaller amounts of other conjugated derivatives are also present. In the case of the glucuronide fraction this could be the hydrocarbon precursor but, as the sulphate yields only a phenolic fraction on hydrolysis, the presence of an additional benzanthrol is indicated.

(b) *The phenolic metabolite*

The phenolic derivative of 1 : 2-benzanthracene isolated from the faeces could not be obtained free from absorbing impurity and this prevented complete characterisation by absorption spectroscopy. In the long wave region above 320 mμ however, bands characteristic of 4′-benzanthrol were present and consistent with this behaviour was the bright golden yellow fluorescence exhibited by the metabolite in sodium hydroxide. In view of the possibility of an additional benzanthrol being present the metabolite was subjected to methylation and chromatographic fractionation in the manner described for methoxybenzpyrenes (Harper, 1958*b*). No marked differences were observed in the spectra of the fractions thus obtained and, on pooling and concentrating *in vacuo*, the absorption spectrum shown in Fig. 2 was recorded. Also shown in Fig. 2 is the spectrum of the phenolic derivative liberated from the sulphate conjugate by mild acid hydrolysis and the two are compared respectively with those of 4′methoxy- and 4′-hydroxy-1 : 2-benzanthracene. The detailed positions of the absorption bands are given in Table I.

TABLE I

Absorption bands in ethanol (mμ)				Absorption bands in hexane (mμ)			
BAX_1	4′–BA–S	BAX_2	4′–BA–G	BA–OH	4′–OH	BA–OMe	4′–OMe
—	—	—	—	226	230	228	231
[246–248]	[244–248]	—	244–246	—	245	246	245–246
252	254	—	254	253	253·5	250–254	253·5
—	—	—	—	[262]	—	—	—
270	272	—	—	[270–272]	—	—	—
280	282	—	[284–286]	[278–280]	277	[280–282]	277
290–291	292	288	288	286	286	287–288	287·5
—	—	—	—	297	297	—	299
—	—	—	308	—	309.5	—	311·5
324	326	—	328	[320–324]	326–327	[326–328]	327
—	—	—	—	337	—	—	—
342	342	[338–342]	342	[340–341]	342	342	342·5
—	—	—	354–356	354–355	353	354	352–353
358	359	[358–362]	358–360	—	359	[358–360]	359–360
—	366–368	—	370	372	371–372	370–372	371
—	—	—	—	—	382	—	381
388	389	392	391–392	394	392	392	391.5

BAX_1 and BAX_2 = X_1 and X_2 metabolites of 1 : 2-benzanthracene.
4′–BA–S and 4′–BA–G = sulphuric (S) and glucuronic (G) esters of 4′–benzanthrol.
BA–OH = free phenolic component of the sulphate conjugate (BAX_1).
BA–OMe = methylated faecal metabolite.
4′–OH and 4′–OMe = free (– OH) and methylated (–OMe) 4′–benzanthrol.

Reference to this data reveals that, above 320 mμ, the spectra of the methylated metabolite and 4′-methoxy-1 : 2-benzanthracene are very similar and is in agreement with the conclusion of Berenblum and Schoental (1943) that hydroxylation of the benzanthracene nucleus occurs in the 4′-position. It will be recalled that this wave length region only was utilised by these workers for their analysis. In the present work however, the full absorption spectra have been recorded and below 320 mμ it is at once apparent that marked differences exist. The major band of 4′methoxy-1 : 2-benzanthracene at 287 mμ is present

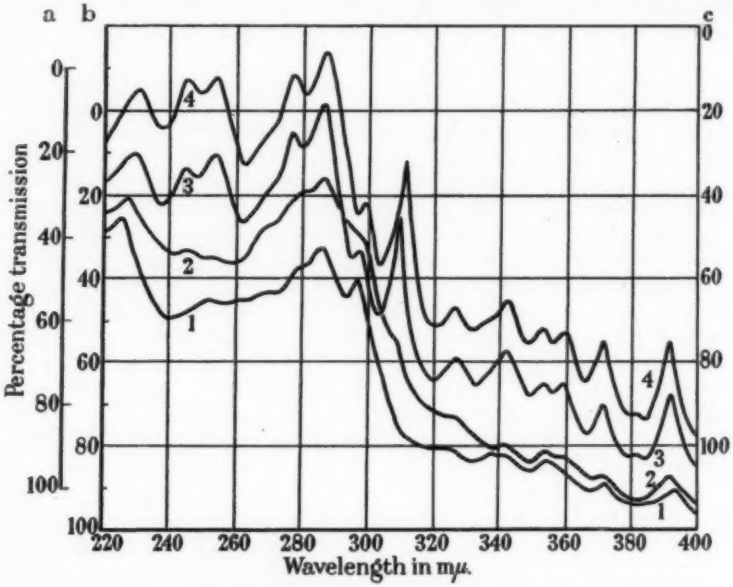


FIG. 2.—Absorption spectra in hexane.

1. Phenolic component of the X_1 type metabolite of benzanthracene (*b* ordinates).
2. Methylated faecal metabolite (*b* ordinates).
3. 4′-benzanthrol (*a* ordinates).
4. 4′-methoxybenzanthracene (*c* ordinates).

in the " metabolic " spectrum but a conspicuous feature is the absence of any band, other than a slight inflection, at 311·5 mμ. The latter is a prominent feature of the 4′- substituted derivatives and is readily detected in all metabolic studies with these compounds. It is doubtful therefore that failure to record this band is due to the presence of masking impurity of natural origin : A possible explanation is that the presence of an additional related methoxylated benzanthracene interferes with the absorption of the 4′-methoxy derivative in this region although chromatographic fractionation revealed no evidence of this. Support for this proposal however is provided by the spectrum of the free phenol liberated from the sulphate conjugate. This is similar to that of 4′-benzanthrol in its general appearance but differences exist in both short and long wave regions. An unknown phenolic derivative with bands at 226, 286, 297, 320–324,

337, 354–355, 372, and 394 mμ would indeed appear to represent the major component of the mixture.

In conclusion, therefore, it can be said that the evidence obtained in this work is consistent with the view that a mixture of phenolic derivatives is formed as a result of the biological hydroxylation of 1 : 2-benzanthracene in the mouse. One of them is 4′-benzanthrol, the other is neither 3-benzanthrol (Jones, 1945) nor 9 : 10-dihydroxy-1 : 2 : -benzanthracene (Berenblum and Schoental, 1943) and is as yet unidentified. By analogy with other hydrocarbons however it may logically be expected to be the 2′-hydroxy derivative.

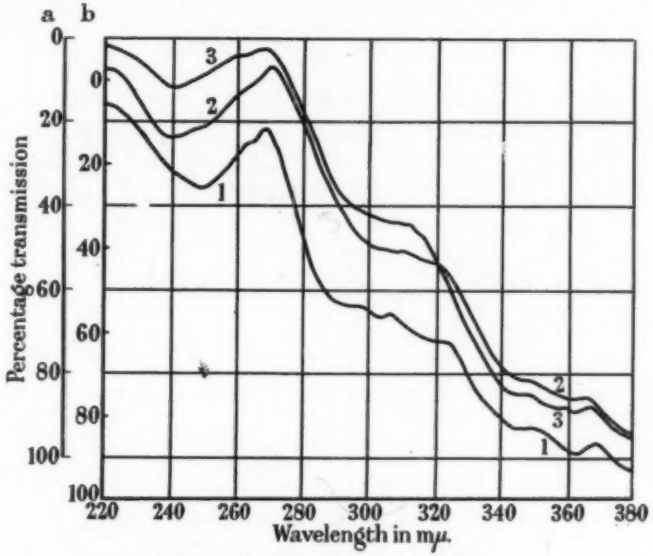


FIG. 3.—Absorption spectra in ethanol.

1. Phenolic metabolite of chrysene (*b* ordinates).
2. X_1 type metabolite of chrysene (*a* ordinates).
3. X_2 type metabolite of chrysene (*a* ordinates).

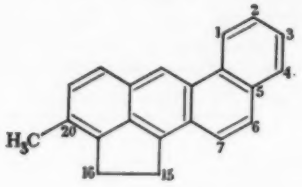
(*B*) *Chrysene*

The phenolic derivative excreted in the faeces after intraperitoneal injection of chrysene in the rat was identified by Berenblum and Schoental (1949) as 3-chrysenol. Attempts to confirm this in the present work with mice have been only partially successful owing to the low yield obtained and difficulty experienced in purification. The experiments have shown however that the two conjugated fractions yield what appears to be the same chrysenol on hydrolysis with takadiastase and β-glucuronidase respectively and that a small amount of free chrysene is also liberated from the glucuronide fraction on hot acid hydrolysis.

The absorption spectra of the metabolites (Fig. 3), although ill-defined, are consistent with a fully aromatic chrysenoid configuration. The conjugated metabolites are therefore concluded to be (3)-chrysenyl sulphate and glucuronide respectively.

(*C*) 20-*Methylcholanthrene*

This hydrocarbon (I) was of particular interest in its divergence from the fully aromatic configurations so far studied. Although existing evidence on the



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metabolism of this compound suggested that hydroxylation occurs on the aromatic benzanthracene nucleus (Cason and Fieser, 1940; Dobriner, Rhoads and Lavin, 1942), general metabolic considerations were in favour of both phenol

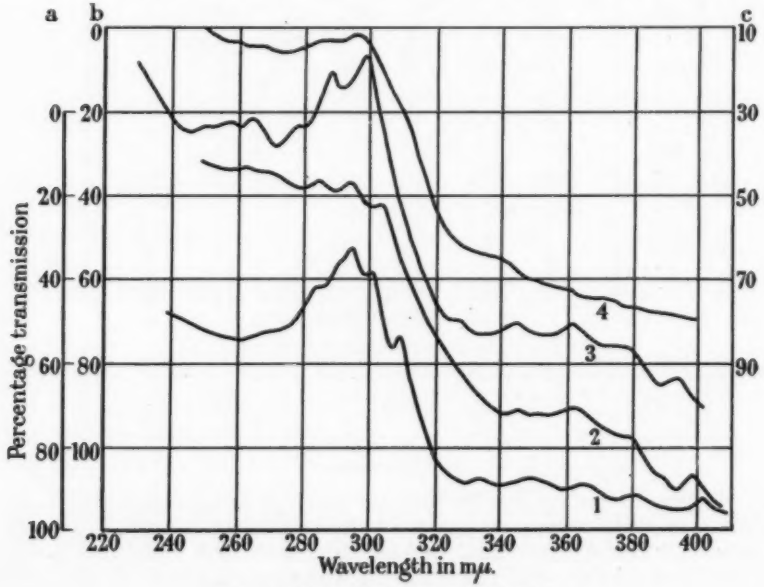


FIG. 4.—Absorption spectra in ethanol.
1. Carboxylic acid metabolite of 20-methylcholanthrene (*a* ordinates).
2. Phenolic metabolite of 20-methylcholanthrene (*a* ordinates).
3. X_1 type metabolite of 20-methylcholanthrene (*b* ordinates).
4. X_2 type metabolite of 20-methylcholanthrene (*c* ordinates).

and carboxylic acid formation. The latter may be expected to arise from the oxidation of the attached methyl group—cf. the metabolism of 2-methylnaphthalene (Grimes and Young, 1956)—or from fission of the 15–16 bond—cf. the

metabolism of acenaphthene (Chang and Young, 1943). This theoretical prediction has been borne out experimentally for two methylcholanthrene derivatives, one phenolic and the other acidic, have been isolated in this work. These were absent from the bile and small intestine but were present in the caecum, large intestine and faeces.

The phenolic derivative possessed normal chromatographic behaviour and the typical fluorescence colour change from blue to yellow occurred on the addition of sodium hydroxide to its ethanolic solution. The absorption spectrum shown in Fig. 4 possessed bands at 284, 294, 304–305, 344–346, [352], 363 [378–380] and 398 mμ. It has not been possible to identify it but, by analogy with other hydrocarbons, it may logically be expected to be either the 2- or 4-hydroxy derivative.

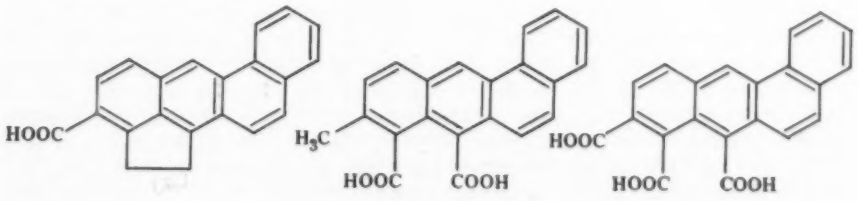
The acidic derivative was not adsorbed on silica gel from xylene or benzene but was tightly held as a narrow blue fluorescent zone at the surface of an alumina column. Elution was achieved with ethanol containing 1 per cent hydrochloric acid, ethanol by itself being ineffective. A solution of the metabolite in this mixture possessed a yellow fluorescence which changed to bright blue on making alkaline with sodium hydroxide. Its acidic nature was further emphasised by its solubility in dilute sodium bicarbonate. In view of these properties the metabolite was concluded to be a carboxylic acid derivative. As the absorption spectrum shown in Fig. 4, with bands at 284, 295, 302, 312, 334, 348–350, 364–366, 378–380 and 402 mμ, establishes the integrity of the aromatic benzanthracene nucleus, three possible structures for the metabolite are :

(*a*) Cholanthrene-20-carboxylic acid (II), arising from oxidation of the methyl group.

(*b*) 6-Methyl-1 : 2-benzanthracene-5 : 10-dicarboxylic acid (III), arising from fission at the 15–16 bond.

or

(*c*) 1 : 2-Benzanthracene-5 : 6 : 10-tricarboxylic acid (IV), arising from a combination of these.



II III IV

If the metabolite is either the di-(III) or tri-(IV) carboxylic acid, then anhydride formation is theoretically possible. This was tested by heating the compound at 200° C. for ten minutes. After cooling and dissolving in ethanol no difference in its chromatographic behaviour and absorption spectrum was observed. A tentative conclusion therefore is that the metabolite is cholanthrene-20-carboxylic acid (II).

The absence of the free phenol and carboxylic acid from the gall bladder and small intestine indicated their formation from the conjugated metabolites present

in these organs. This was confirmed on enzymatic hydrolysis of the conjugates when the sulphate fraction yielded free phenol alone whilst the glucuronide fraction yielded a mixture of phenol and carboxylic acid. In the latter case advantage was taken of the differential solubilities of the derivatives in sodium bicarbonate to effect a ready separation of the two components from solution in ether. The same behaviour was observed on acid hydrolysis under nitrogen but a further phenomenon recorded in this instance was the liberation of a trace amount of 20-methylcholanthrene from the glucuronide fraction.

The absorption spectra of the conjugated fractions, shown in Fig. 4, establish that the aromatic benzanthracene nucleus is intact. It is concluded from this work therefore that the X_1 type metabolite is a sulphuric acid ester of a phenolic derivative of 20-methylcholanthrene whilst the X_2 type contains a mixture of glucuronic acid esters of the same phenol and cholanthrene-(20)-carboxylic acid together with an acid-decomposable precursor of the hydrocarbon. Indications of the presence of other water soluble metabolites have also been obtained but these have not been pursued further.

(*D*) 1 : 2 : 5 : 6-*Dibenzanthracene*

A phenolic derivative, 4′ : 8′-dihydroxy-1 : 2 : 5 : 6-dibenzanthracene, was the first metabolite of 1 : 2 : 5 : 6-dibenzanthracene to be identified (Cason and Fieser, 1940; Dobriner, Rhoads and Lavin, 1942). Subsequent investigation by Heidelberger and his collaborators in the United States established that the metabolism of this hydrocarbon is accompanied by extensive degradation of the aromatic nucleus (Heidelberger and Jones, 1948; Heidelberger, Kirk and Perkins, 1948) and consistent with this finding is the low yield of fluorescent material which has been isolated in the present work. Owing to the small amounts of metabolites obtained it has not been possible to characterise the compounds individually by absorption spectroscopy although a mixture of the conjugated derivatives extracted from bile possessed absorption indicative of an intact dibenzanthracene nucleus.

Enzymatic hydrolysis of the two conjugated fractions as described resulted in the liberation of a phenolic derivative as adjudged by the fluorescence colour change from blue to yellow occurring on the addition of sodium hydroxide. It was concluded from this behaviour therefore that a phenolic derivative, presumably 4′ : 8′-dihydroxy-dibenzanthracene, is excreted via the bile in conjugation with sulphuric and glucuronic acids. The conjugates could not be detected within the liver or kidney and were absent from the caecum and large intestine. Free phenol was present in the latter organs, however, suggesting that hydrolysis of the conjugates occurs at this site.

These findings are supported by the data reported by Heidelberger, Kirk and Perkins (1948) on the excretion of ^{14}C-labelled dibenzanthracene following intravenous injection in the mouse. The extraction procedure adopted by these workers enabled the radioactivity of the material under investigation to be split into four fractions which they considered to contain respectively :

(1) Unchanged hydrocarbon.

(2) Unconjugated material.

(3) Extremely water soluble organic substances together with some less soluble material which was conjugated with water solubilising groups.

(4) A solid residue which was partly soluble in water.

Analysis of the data obtained respectively from bile and faeces shows that, during passage through the intestine, there is a transfer of radioactivity from fraction 3, the major component of the biliary activity, to fractions 2 and 4. The increase in fraction 2 is most pronounced, from 0·7 per cent in the bile to 14 per cent in the faeces, suggesting that unconjugated derivatives in the faeces are derived from conjugated compounds excreted in the bile.

It was further shown by these workers that fraction 2 of the faeces contains a mixture of phenolic, acidic and neutral components so that the same sequence of conjugation and hydrolysis presumably occurs with the dicarboxylic acid degradation products as has been established for cholanthrene-(20)-carboxylic acid in the present work.

(*E*) *Anthracene*

The metabolism of anthracene was studied by Boyland and Levi (1935, 1936*a*, 1936*b*) who reported the excretion in the urine of a perhydroxylated derivative, 1 : 2-dihydroxy-1 : 2-dihydro-anthracene, both free and conjugated with glucuronic acid, an unidentified acid-decomposable precursor of the hydrocarbon and 1-anthrylmercapturic acid. The latter has since been shown by Knight and Young (1958) to arise during extraction from the action of mineral acid upon a precursor designated by the general term " premercapturic acid ".

The excretion of metabolites in the bile has also been reported (Chalmers and Peacock, 1941 ; Chalmers, 1957) but these have not been identified.

The present experiments with anthracene have shown that X_1 and X_2 type derivatives, analogous to those yielded by the other hydrocarbons studied in this series, are not formed during the metabolism of this compound. The isolation of such compounds, i.e. 1-anthryl sulphuric and glucuronic acids, from the bile following injection of 1-anthrol, under the same conditions of extraction, established that failure to detect these derivatives during metabolism of the parent hydrocarbon was not due to defects of the extraction process.

The situation was found to be quite different however when the bile and aqueous extracts of the duodenum and small intestine were first subjected to mild acid hydrolysis. Under these conditions an X_2 type derivative, identical with 1-anthrylglucuronic acid, and free anthracene were readily extractable from the acid solutions. A blue fluorescent chloroform soluble fraction was also present but this was not investigated further.

As the 2-hydroxy-1 : 2-dihydro- 1 -anthrylglucuronic acid excreted in the urine was found by Boyland and Levi (1936*a*) to break down readily under the influence of acid to yield 1-anthrylglucuronic acid, it was concluded from the above behaviour that this perhydroxylated conjugate is also excreted in the bile together with an acid-decomposable precursor of anthracene and possibly a " premercapturic acid ".

DISCUSSION

The investigations reported in this and previous publications were part of a programme designed to determine the role, if any, played by the metabolism of polycyclic hydrocarbons in induced carcinogenesis. Should the formation of the metabolites be implicated in the carcinogenic mechanism or themselves be the initiators of it—cf. the mode of action of 2-naphthylamine (Bonser, Clayson,

Jull and Pyrah, 1952)—it was considered necessary that the following requirements be fulfilled :

(*a*) They should be present within the tissues of the body where the carcinogenic effect is applied.

(*b*) The same type of metabolites should be formed from all carcinogenic but not non-carcinogenic hydrocarbons (quantitative factors are ruled out by the fact that the carcinogenic members exert their effect when present in only trace amounts).

(*c*) If themselves the proximate carcinogenic agents then this activity should be detectable by normal laboratory methods of testing.

Requirement (*a*) was met by the intermediate X_1 and X_2 metabolites of 3 : 4-benzpyrene previously isolated by Weigert and Mottram (1943). Extension of this work to a range of hydrocarbons has now shown that similar intermediates are formed from the non-carcinogenic pyrene, the weakly carcinogenic chrysene and 1 : 2-benzanthracene and the strongly carcinogenic 1 : 2 : 5 : 6-dibenzanthracene and 20-methylcholanthrene. These intermediates have been found to possess a common structure, i.e. phenolic derivatives conjugated with sulphuric acid (X_1) and glucuronic acid (X_2), and the association of acid-decomposable precursors of the hydrocarbons with the X_2 fractions suggests that these too may contain a glucuronide conjugated hydroxyl group. Indeed, the experiments with pyrene (Harper, 1957*a*, 1958*a*) suggest that, in like manner to the phenolic conjugates, the glucuronide moiety of the precursors is split off during excretion for the pyrene precursor in the faeces was then found in association with the neutral quinone fraction on the chromatogram. Consistent with this behaviour would be the presence of an $\alpha : \beta$-dihydro-α-hydroxy configuration similar to that established for the precursor of naphthalene (Boyland and Solomon, 1955).

The metabolisms of 1 : 2 : 5 : 6-dibenzanthracene and 20-methylcholanthrene are further complicated by the formation of carboxylic acid derivatives but this does not appear to be a general feature of the carcinogenic series.

The anomalous behaviour of anthracene must be attributed to the exclusive process of perhydroxylation, as opposed to that of hydroxylation, operating on this hydrocarbon. As dihydrodiols appear to undergo conjugation exclusively with glucuronic acid the failure of anthracene to bring about any significant increase in the level of ethereal sulphate excretion in the urine (Elson, Goulden and Warren, 1945) is consistent with the finding that phenol formation does not occur.

Only in the case of 3 : 4-benzpyrene has it been possible to isolate sufficient of the intermediate metabolites for carcinogenicity testing. These have been tested singly, in combination with each other and in combination with a co-carcinogen, croton oil, but in no case has malignant tumour formation been observed (Harper, 1957*b*, 1958*d*). Other hydrocarbon derivatives tested for activity include the hypothetical metabolites of anthracene, 1- and 2-anthrol. These were found to be inactive suggesting that the non-carcinogenic nature of this hydrocarbon is not due to the protective influence of dihydrodiol formation (unpublished data).

The overall conclusion from this work therefore is that there appears to be no specific difference in the chemical nature of the metabolites derived respectively from carcinogenic and non-carcinogenic hydrocarbons. It is unlikely then that

the known metabolites represent the proximate carcinogenic agents and this is supported experimentally both by the negative findings of carcinogenic activity referred to above and by the negative findings reported for phenolic and acidic metabolites by other workers (e.g. Hartwell, 1951; Heidelberger and Wiest, 1951; Allen, Boyland and Watson, 1956).

Conversely, the formation of similar metabolites from both carcinogenic and non-carcinogenic hydrocarbons would appear to provide no indication of an association of metabolism with carcinogenesis. However, one important difference not yet referred to lies in the position of the hydrocarbon molecule at which biochemical hydroxylation takes place. This is exemplified by reference to Table II in which existing data on the chemical reactivities and metabolic hydro-

TABLE II

Hydrocarbon	Reactive positions	Reactive bonds	Metabolic positions (phenols)	Metabolic bonds (dihydrodiols)	Carcinogenic activity
Naphthalene	1 (2)	1–2	1 (2)	1–2	–
Anthracene	9, 10	1–2 3–4	(9, 10)	1–2	–
Phenanthrene	9, 10	9–10	..	9–10 (1–2)	–
Pyrene	3	1–2	3	..	–
Chrysene	2	1–2	3	..	+
1 : 2-Benzanthracene	9, 10	3–4	4′	..	+
9 : 10-Dimethyl-1 : 2-benzanthracene	9, 10	3–4	4′	..	+
1 : 2 : 5 : 6-Dibenzanthracene	9, 10	3–4 7–8	4′, 8′ 2′, 6′	..	+
3 : 4-Benzpyrene	5 (10)	6–7	8, 10, F_1 (5, 5 : 8, 5 : 10)	..	+
20-Methylcholanthrene	15 (11, 14)	6–7	2 or 4? (not 15)	..	+

Numbers in brackets refer to positions of secondary activity.

xylation of a range of hydrocarbons is summarised. The non-carcinogenic members, it will be seen, are characterised by a tendency to undergo either phenol or dihydrodiol formation, the former occurring at the reactive positions of the molecule and the latter at the reactive bonds. The carcinogenic members on the other hand are typified by a tendency to undergo phenol formation only and this occurs, in general, at positions of the molecule which are inert towards chemical attack. In order to account for this fact that carcinogenic hydrocarbons are subjected to hydroxylation in these normally inert positions it has been suggested (Dickens and Weil-Malherbe, 1945; Boyland, 1948, 1950; Berenblum and Schoental, 1949) that the reactive centres of the carcinogen are initially blocked by cellular constituents and that, in this bound state, the "metabolic positions" become activated. An alternative possibility, however, is that different hydroxylating mechanisms are operative in the metabolism of carcinogenic and non-carcinogenic hydrocarbons. It is hoped to discuss both these possibilities in greater detail in a succeeding paper. At present it is sufficient to say that any association of a known metabolic process with carcinogenesis most probably lies within or before the primary stage of aromatic hydroxylation.

SUMMARY

1. Previous studies of the intermediary metabolism of pyrene and 3 : 4-benzpyrene have been extended to a range of hydrocarbons, namely 1 : 2-benzanthracene, chrysene, 20-methylcholanthrene, 1 : 2 : 5 : 6-dibenzanthracene and anthracene. All of these, with the exception of anthracene, have been found to undergo hydroxylation to phenolic derivatives. These are excreted mainly via the bile in conjugation with sulphuric and glucuronic acids and hydrolysis then occurs during passage through the caecum and large intestine. Consequently it is the free phenols that are detected when investigations are confined to the faeces alone.

2. Acid-decomposable precursors of the hydrocarbons have been detected in association with the glucuronide fraction on the chromatogram. The presence of a glucuronide moiety in the precursors is therefore indicated and experiments with pyrene suggest that, in like manner to phenolic conjugates, this is split off during excretion.

3. Evidence has been obtained that 1 : 2-benzanthracene is subjected to hydroxylation in two positions of the molecule, the 4′- and possibly, by analogy with other hydrocarbons, the 2′-.

4. 20-Methylcholanthrene has also been found to yield a carboxylic acid derivative, provisionally identified as cholanthrene-20-carboxylic acid, and this undergoes the same sequence of glucuronide conjugation and hydrolysis during excretion as has been established for the phenolic metabolite. The data reported on the metabolism of 1 : 2 : 5 : 6-dibenzanthracene-9 : 10-^{14}C by Heidelberger, Kirk and Perkins (1948) is considered to be consistent with a similar behaviour for the dicarboxylic acid derivatives yielded by this hydrocarbon.

5. It is suggested that the anomalous behaviour of anthracene is due to the exclusive process of perhydroxylation operating on this hydrocarbon. The metabolites excreted in the bile have not been isolated but their behaviour towards mild acid hydrolysis identifies them as 2-hydroxy-1 : 2-dihydro-1-anthryl glucuronic acid and an acid decomposable precursor of the hydrocarbon.

6. It is concluded from these studies that the only important difference between the known metabolisms of carcinogenic and non-carcinogenic hydrocarbons lies in the positions of the molecule at which hydroxylation initially occurs. Consequently any association of metabolism with carcinogenesis most probably lies either within or before this stage.

The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

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THE POLYCYCLIC HYDROCARBONS: METABOLISM, CELLULAR BINDING AND CARCINOGENESIS

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Received for publication October 31, 1959

THE data reported in the preceding communication (Harper, 1959) have led to the conclusion that the only important difference between the metabolisms of carcinogenic and non-carcinogenic hydrocarbons lies in the positions of the molecule at which hydroxylation initially occurs. Two possible explanations of this difference were considered to be :

(*a*) That the reactive centres of carcinogenic hydrocarbons are initially blocked by cellular material ;

or

(*b*) that different mechanisms of hydroxylation are operative during the metabolism of carcinogenic and non-carcinogenic hydrocarbons respectively.

The mechanism of aromatic hydroxylation is a subject which has received much attention in recent years but so far the investigations have been confined to only two members of the polycyclic hydrocarbon series, namely, naphthalene and 3 : 4-benzpyrene. Fortunately for the purpose of this discussion, however, these may be regarded as typical members of the non-carcinogenic and carcinogenic hydrocarbons respectively.

It was first shown by Mitoma, Posner, Reitz and Udenfriend (1956) that an enzyme system capable of hydroxylating a variety of organic compounds was able to effect the *in vitro* conversion of naphthalene into 1-naphthol (but not 2-naphthol) and a dihydrodiol-like compound which yielded 1-naphthol on acid hydrolysis. The system was located in the microsomal fraction of liver homogenate (but not of brain, kidney, lung and muscle) and required reduced triphosphopyridine nucleotide and oxygen for activity.

A similar enzyme system was found by Conny, Miller and Miller (1957) to effect the hydroxylation of 3 : 4-benzpyrene and the products thus obtained were identical to those yielded during *in vivo* metabolism of the hydrocarbon. The " hydroxylase " was similarly located in the liver microsomes and, although both reduced tri- and diphosphopyridine nucleotides (TPNH and DPNH) and oxygen were required for maximal activity, high activity was supported by TPNH and oxygen alone. The system differed from that of Mitoma *et al.* (1956) however in not being inhibited by the metal binding compounds α, α'-dipyridyl and *o*-phenanthroline.

Further investigation of the naphthalene hydroxylating system by Booth and Boyland (1957, 1958) confirmed the findings of Mitoma *et al.* with respect to the cellular distribution and requirements of the system but it was found

that neither α, α'-dipyridyl nor *o*-phenanthroline inhibited activity to any great extent. Also, in like manner to the benzpyrene hydroxylating system, inhibition was not observed with either cyanide or cysteine but strong inhibition occurred in the presence of *p*-chloromercuribenzoate suggesting the involvement of sulphydryl groups.

On this evidence therefore it appears probable that the same enzyme, or closely related enzymes with the same co-enzyme requirements, are responsible for the hydroxylation of naphthalene, a non-carcinogen, and 3 : 4-benzpyrene, a potent carcinogen. If such is the case it is unlikely that such closely related systems operate by different mechanisms.

This brings us back to the alternative possibility therefore, of an initial blocking of the reactive centres of the carcinogen by cellular material. The problem in this case is to determine at what region of the molecule such binding is likely to occur, for reference to Table II of the preceding communication (Harper, 1959) reveals the presence of both reactive carbon atoms and reactive bonds although these coincide in certain cases.

The possibility of binding across reactive non-adjacent carbon atoms was considered by Dickens and Weil-Malherbe (1946) who compared metabolic hydroxylation with the chemical method used most successfully in the synthesis of certain metabolic phenols, i.e. sulphonation of the meso-quinone followed by reduction and alkali fusion. An attempted synthesis of 8-benzpyrenol starting with the 5 : 10-quinone was unsuccessful owing to failure at the reduction stage but none the less Dickens and Weil-Malherbe considered that this chemical evidence tallies well with the conception that the most reactive centres of the hydrocarbon molecule are blocked with a cellular constituent and that oxidation then occurs at the most reactive centres remaining.

The other possibility of binding at the reactive bond of the hydrocarbon was proposed by Boyland (1948, 1950*a*) and a similar conclusion concerning the formation of 3-chrysenol from chrysene was arrived at by Berenblum and Schoental (1949). The reactive bond corresponds, in most cases, to the so-called K-region of the molecule and great significance is attached to this proposal when considered in relation to the evidence obtained by the Heidelberger school on the binding of hydrocarbons to protein within the skin. In the case of one hydrocarbon, 1 : 2 : 5 : 6-dibenzanthracene, it has actually been established that about 25 per cent of the bound total is attached through both mono- and di-amido linkages in one K-region (Bhargava and Heidelberger, 1956) so that here is experimental evidence in support of the Boyland hypothesis.

A theoretical treatment of this problem by Pullman and Pullman (1955) was based upon the somewhat arbitrary assumption that the hydrocarbon, bound through its K-region, then exists in an ortho-quinonoidal configuration. In the example selected, 1 : 2-benzanthracene, it was calculated that the reactive centre of the hydrocarbon bound in this way then resides on the 3′ carbon atom and not the metabolic 4′ position. This difficulty was overcome by postulating the formation of an epoxide across the 3′–4′ bond which then undergoes hydrolysis under enzymatic control to yield the 3′–4′ dihydrodiol. Finally the elements of water are split off leaving the hydroxyl group in the 4′ position.

Despite the obvious limitations of such a hypothetical treatment it will be seen that both these proposals, one involving a para and the other an ortho form of binding, are unable in themselves to account for the formation of more

than one phenol from the same hydrocarbon. Indeed, the mechanism proposed by Pullman and Pullman (1955) leads to the formation of the same phenol as that obtained from the para-quinone by the described chemical synthesis. Examples of phenolic metabolites are shown in Fig. 1 and it will be seen that

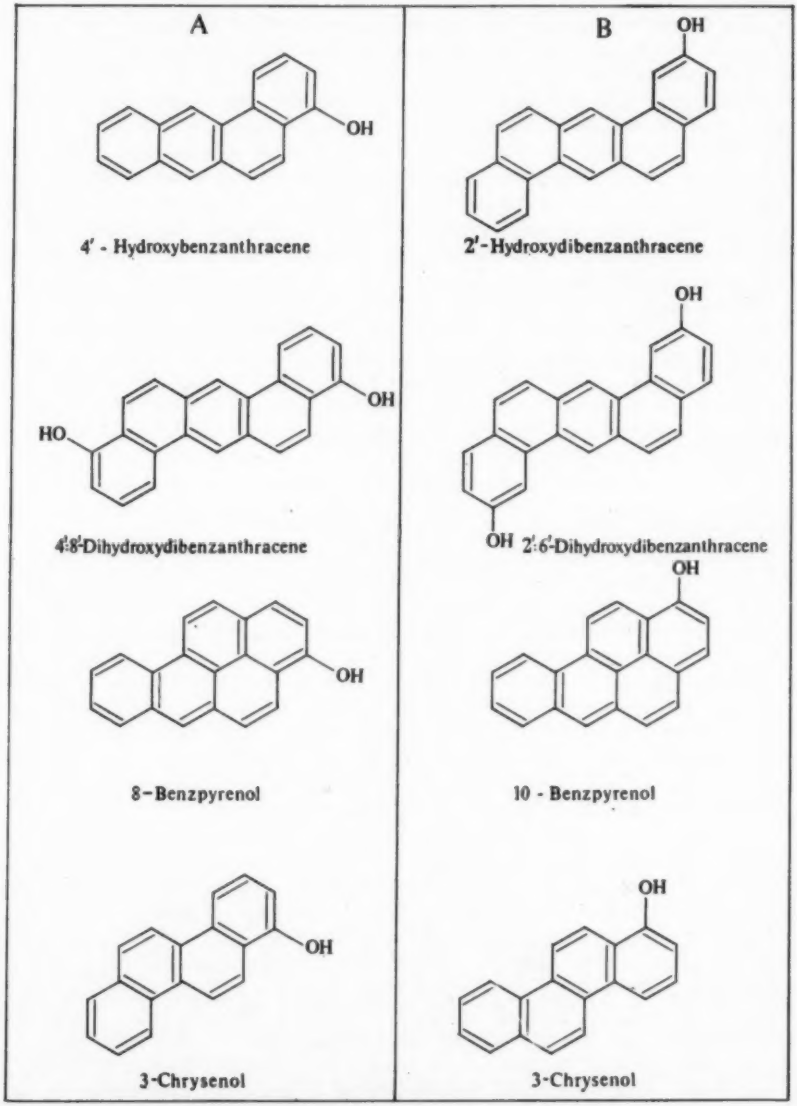


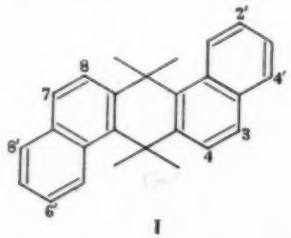
FIG. 1.—Phenolic metabolites of the polycyclic hydrocarbons.

these can be classified on the basis of the structural relationship they bear to one another into two distinct groups designated as A and B in the diagram. What is proposed by the author therefore is that the hydrocarbon may undergo both ortho and para forms of binding during hydroxylation and that in these two states different secondary positions of the molecule become activated. Such a proposal is not entirely speculative for only 25 per cent of the total bound 1 : 2 : 5 : 6-dibenzanthracene could be accounted for by binding in the K-region (Bharagava and Heidelberger, 1956). The remaining 75 per cent is presumably bound at some other region of the molecule. Also 1 : 2 : 3 : 4-dibenzanthracene does not possess an active K-region and yet is bound to an even greater extent than the 1 : 2 : 5 : 6-isomer (Heidelberger and Moldenhauer, 1956). In this case evidence of binding in the reactive meso-positions has in fact been obtained (Oliverio and Heidelberger, 1958).

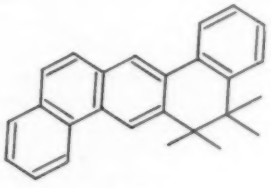
The essential problem then is to determine which form of binding is responsible for the formation of Group A phenols and which form for Group B. For this purpose the effect of both forms of binding will be considered in relation to the products yielded by individual hydrocarbons. As the exact mode of binding is as yet unknown the hydrocarbon will be assumed to form an addition type of complex as proposed by Boyland (1950*a*). In such compounds the nuclear bond system is that of the ortho- or para-quinone although there is little contribution of the bond linkages to the resonance energy of the system. Should the binding prove to be of a different nature however, it is anticipated that similar considerations will apply.

1 : 2 : 5 : 6-*Dibenzanthracene*

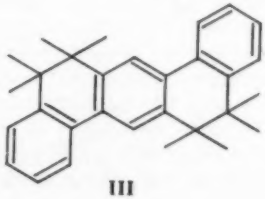
Three phenolic metabolites of this hydrocarbon have now been identified, the 4′ : 8′-dihydroxy derivative from the rat and mouse (Cason and Fieser, 1940) and the 2′-hydroxy and 2′ : 6′-dihydroxy derivatives from the rabbit (Labudde and Heidelberger, 1958). In this instance where the molecule is symmetrical about a central axis, it is to be anticipated that binding across this central axis (I) will assure equal activation in the two halves of the molecule



I



II

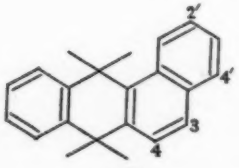


III

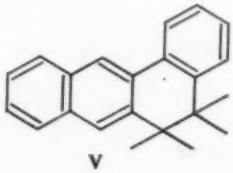
(cf. the sulphonation of 1 : 2 : 5 : 6-dibenz-9 : 10-anthraquinone). Monohydroxylation would not therefore be expected. If on the other hand, binding were to occur at one of the two K-regions (II) then in this instance, where the activating group is asymmetrically situated, it is unlikely that two positions of the molecule would become activated to the same extent. Consequently the formation of a monohydroxylated derivative only would be favoured. In the event of binding at both K-regions, however, the activation in both halves of the molecule would then be equal and dihydroxylation would most probably take place.

It will be seen therefore that the formation of both mono- and di-hydroxylated products in the rabbit is best explained on the basis of an ortho form of binding at one and both K-regions respectively. Conversely the formation of a dihydroxylated—but not a monohydroxylated—derivative in the rat and mouse is consistent with a para form of binding across the reactive meso-positions. The inference is therefore that species differences in metabolism are due to differences in the region of the molecule at which linkage to cellular material occurs during hydroxylation in these species. If this reasoning is correct an ortho form of binding would appear to be favoured in the rabbit and a para form in the rat and mouse. The question immediately arises, however, as to why the mouse does not yield a mixture of phenolic metabolites for K-region binding in this species is now an experimentally established fact. One obvious answer is that the 2′- and 2′ : 6′-hydroxylated compounds are formed but in amounts so small that they have so far escaped detection. This behaviour would then fall into line with that of 3 : 4-benzpyrene for which only a quantitative difference in metabolism has been reported (see later). A possible explanation of this is that the bound complex II behaves as a 2 : 3-disubstituted phenanthrene derivative and as such is particularly susceptible to oxidation at the reactive 9–10 bond, corresponding to the 7–8 bond of dibenzanthracene. Consistent with this hypothesis is the formation within the tissues of the 3 : 4-quinone (Heidelberger, Hadler and Wolf, 1953) and 2-phenylphenanthrene-3 : 2′-dicarboxylic acid (Bhargava, Hadler and Heidelberger, 1955).

1 : 2-*Benzanthracene*



IV



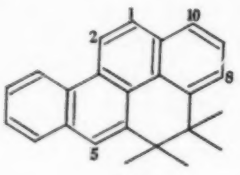
V

Application of this reasoning to 1 : 2-benzanthracene leads to the conclusion that, by analogy, the 4′-hydroxy metabolite, a Group A phenol, is formed as a result of binding across the reactive meso-positions (IV). The formation of 2′-hydroxy-1 : 2-benzanthracene is therefore to be anticipated as a consequence of binding in the K-region (V) and it is significant that the evidence reported in the preceding publication is consistent with the formation of an additional phenolic metabolite. It would obviously be of great interest to determine the major site of metabolism in the rabbit where, according to theory, a K-region

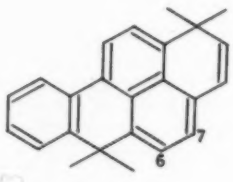
binding of hydroxylation is favoured. Investigations are already in hand to test this proposal.

3 : 4-*Benzpyrene*

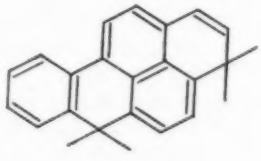
The formation of six different phenolic metabolites from 3 : 4-benzpyrene has now been reported, 5-benzpyrenol (Pihar and Spáleneý, 1956), 8-benzpyrenol (Berenblum, Crowfoot, Holiday and Schoental, 1943), 10-benzpyrenol (Berenblum and Schoental, 1946), 5 : 8- and 5 : 10-dihydroxy-benzpyrenes (Conney *et al.*, 1957) and an unidentified phenol designated as the F_1 metabolite (Weigert and Mottram, 1946 ; Harper, 1958).



VI



VII



VIII

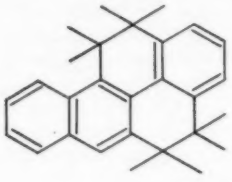
The ease with which 3 : 4-benzpyrene undergoes oxidation to a mixture of 5 : 8-, 5 : 10-, and possibly 6 : 7-quinones (Cook and Schoental, 1950) suggests that the molecule readily assumes the nuclear bond structures VI–VIII. It is possible then that binding may occur across the 5 : 8-, 5 : 10-, and 6 : 7- (K-region) positions of the molecule, each of which leads to the formation of a different phenol. By analogy with 1 : 2 : 5 : 6-dibenzanthracene an ortho form of binding in the K-region (VI) may be expected to activate the 10-position and it is significant that 10-benzpyrenol, a Group B phenol, is formed to a greater extent in the rabbit than in the rat and mouse (Berenblum and Schoental, 1946).

As the para configuration of structure VII is analogous to that of a true meso-quinone it is then possible that binding across the 5,10 positions would result in activation of the 8-position (Group A). By elimination, formation of the F_1 metabolite may then be attributed to binding in the 5,8 positions.

An alternative treatment, however, is to regard the K-region bound structure VI as a 6 : 7-disubstituted chrysene. On this interpretation position 2 of the chrysene molecule, corresponding to the 1-position of benzpyrene, would be expected to be most active and indeed may be expected to represent a major site of hydroxylation. It is possible therefore that the F_1 derivative is 1-benzpyrenol and consistent with this hypothesis is the fact that F_1 is undoubtedly

the major product of hydroxylation during the first few hours following injection of 3 : 4-benzpyrene (Weigert and Mottram, 1946 ; Harper, 1958).

If structure VI does in fact exhibit the normal chemical reactivity of chrysene a further stage of binding may occur at the reactive 1–2 bond (IX) and, by analogy with chrysene (see later), lead to the formation of 10-benzpyrenol.

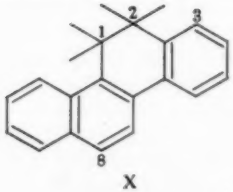


IX

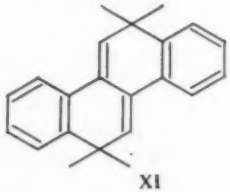
The other phenolic metabolites of benzpyrene, 5-benzpyrenol and the 5 : 8- and 5 : 10-dihydroxy derivatives, differ from the others so far considered in that hydroxyl groups have entered the molecule in the chemically reactive positions. A possible explanation of this is that hydroxylation may occur to a certain extent when the hydrocarbon is in an unbound state. Perhaps significantly the latter compounds have only been detected in *in vitro* studies with the isolated microsomal system in which the normal *in vivo* pattern of cellular binding may not prevail to the same extent.

Chrysene

The hydroxylation of chrysene is more difficult to interpret for the 3-chrysenol rat metabolite (Berenblum and Schoental, 1949) may be regarded as either a Group A or Group B phenol. By analogy with other hydrocarbons an ortho form of binding in the K-region (X) must be favoured in this instance. A para form of binding across the reactive 2,8 positions (XI) on the other hand may be expected to give rise to a symmetrical dihydroxylated derivative.

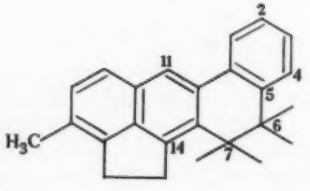


X

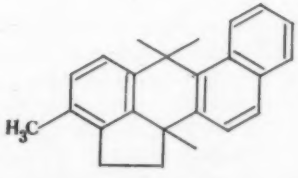


XI

20-*Methylcholanthrene*



XII

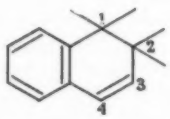


XIII

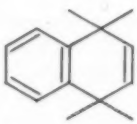
The two possible modes of binding for 20-methylcholanthrene are shown in XII and XIII. By analogy with other hydrocarbons these may be expected to activate the 2 and 4 positions respectively. The ortho bound structure, XII, however, may be regarded as a disubstituted acenaphthene derivative and as such would be more prone to oxidation at the 15–16 bond of the pentacyclic ring (cf. the metabolism of acenaphthene, Chang and Young, 1943). The evidence reported in the preceding paper suggests that this does not occur to any appreciable extent and a para form of binding (XIII) across the 11, 14 positions is therefore favoured. A possible structure for the phenolic metabolite isolated in that work is therefore 4-hydroxy-20-methylcholanthrene.

So far these theoretical considerations have been confined to the carcinogenic members of the polycyclic hydrocarbons. As was pointed out in the preceding publication (Harper, 1959) the products obtained from non-carcinogenic hydrocarbons are consistent with the view that hydroxylation occurs at the most reactive positions of the molecule and perhydroxylation at the most reactive bonds although the perhydroxylation of phenanthrene at the 1–2 bond in the rabbit is an obvious exception to this generalisation. This behaviour may be interpreted as indicating that the non-carcinogenic members are metabolised primarily when in an unbound state and would be in accordance with the conclusion of Heidelberger and Moldenhauer (1956) that non-carcinogenic hydrocarbons do not undergo binding to protein to any appreciable extent. The evidence of Hadler, Darchun and Lee (1957), however, would suggest that the non-carcinogens may go through a transient protein-bound phase of at least the same order of magnitude as that observed with the carcinogens. The findings of Calcutt (1958) are not only consistent with this view but also show that binding may occur in other tissues apart from the skin. It is of interest therefore to speculate upon the effects of ortho and para forms of binding on the reactivities of this class of compounds.

Naphthalene



XIV

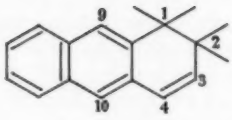


XV

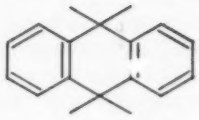
A variety of hydroxylated products, 1- and 2-naphthol (Bourne and Young, 1934; Corner and Young, 1954, 1955), 1 : 2-dihydroxy-naphthalene (Corner and Young, 1954, 1955), 1 : 2-dihydroxy-1 : 2-dihydro-naphthalene (Young, 1947) and 1-hydroxy-1 : 2-dihydro-naphthalene (Boyland and Solomon, 1955) are formed during the metabolism of this hydrocarbon. In all cases, however, attack is at the 1–2 bond and any form of binding must therefore activate this region of the molecule.

The two possible modes of binding for naphthalene are shown in XIV and XV. It is highly improbable that either of these lead to activation in the unsaturated benzene ring and the only possible means of activation of the metabolic bond is therefore via the ortho-bound structure XIV, as proposed by Boyland (1950*b*).

Anthracene



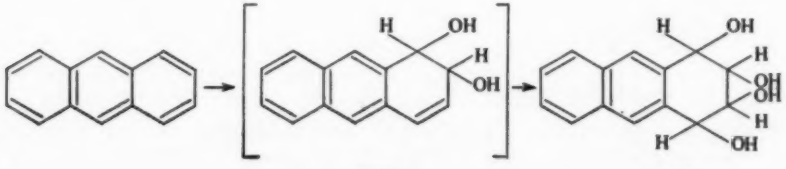
XVI



XVII

Unlike naphthalene the reactive carbon atoms of anthracene do not coincide with the reactive bond. If, as concluded earlier, hydroxylation of the unbound hydrocarbon occurs at the reactive positions of the molecule then the derivative 9 : 10-dihydroxyanthracene (or 9 : 10-anthraquinone) is to be anticipated as a result of this process. There is in fact evidence that the 9 : 10-quinone is excreted as a metabolite of anthracene in the urine (Boyland and Levi, 1936) although the possibility of this being present as an impurity in the administered hydrocarbon was not discounted in that work. Since then however it has been reported that administered 9 : 10-anthraquinone is itself subjected to hydroxylation (Sato, Fukuyama, Yamada and Suzuki, 1956) so that the quinone isolated by Boyland and Levi was probably a true product of hydroxylation and not due to impurity. The major point of attack, however, is at the 1–2 bond resulting in the formation of 1 : 2-dihydroxy-1 : 2-dihydro-anthracene (Boyland and Levi, 1935). What must be decided, therefore, is whether the ortho or para bound structures (XVI and XVII) above are characterised by any marked reactivity at the 1–2 bond.

Unfortunately little is known of the reactivity of the 1,2 addition compounds although the fact that the perhydroxylation of anthracene with Criegie's reagent (Cook and Schoental, 1948) leads to the formation of the 1 : 2 : 3 : 4-tetrahydroxy-1 : 2 : 3 : 4-tetrahydro derivative (XVIII) and not the 1 : 2-dihydrodiol (XIX) suggests that marked activation of the 3–4 bond may occur, assuming the course of the reaction to be as shown.



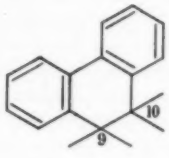
XIX XVIII

The process of para-blocking in the 9,10 position, however, as with hydrogenation and quinone formation, leads to the formation of particularly stable compounds in which the reactivity of the molecule as a whole is greatly reduced by comparison with that of anthracene. Addition at these points therefore is to be regarded as a deactivating rather than activating mechanism.

An ortho form of binding (XVI), as proposed by Boyland and Wolf (1950), is therefore favoured in this instance although the alternative para form is not entirely discounted.

Phenanthrene

Phenanthrene is the sole non-carcinogenic hydrocarbon for which a species difference in the site of hydroxylation has been reported, the 9 : 10-dihydrodiol being formed in the rat and mouse and a mixture of the 9 : 10- and 1 : 2-dihydrodiols in the rabbit (Boyland and Wolf, 1948, 1950). The fact that the carbon atoms comprising the highly reactive 9–10 bond are themselves the reactive positions makes it unlikely that perhydroxylation at this bond is due to binding at some other region of the molecule.



XX

The additional formation of the 1 : 2-dihydrodiol in the rabbit, however, may be attributed to binding across the reactive 9–10 bond (XX), as proposed by Boyland and Wolf (1950), and provides further support for the suggestion that an ortho form of binding is favoured in the rabbit. The fact that 9 : 10-dihydrophenanthrene undergoes Friedel and Crafts' acylation exclusively in the 2-position is evidence that such addition may have a directive influence.

To summarise these proposals, therefore, it can be said that the metabolism of non-carcinogenic hydrocarbons is best explained on the assumption that hydroxylation occurs when they are either in a free state or bound to cellular material through their most reactive bond. The metabolism of carcinogenic members on the other hand would suggest that hydroxylation occurs principally after binding and the formation of different phenols from the same hydrocarbon is then explainable if it is postulated that binding may occur either at the reactive bond or reactive positions of the molecule. On such reasoning it is tentatively concluded that species differences in the site of hydroxylation are due to differences in the relative contributions of these distinct forms of binding to the total amount bound in any one species. An ortho form of binding at the reactive bond would appear to be favoured in the rabbit and a para form across the reactive positions in the rat and mouse.

A critical test of these proposals is the verification that the reactive centres of the ortho and para addition products, or perhaps those of the derived ortho- and para-quinonoidal configurations, do in fact correspond to those attacked during metabolism. Furthermore, as the binding data referred to in this discussion has been obtained from skin, not only must it be established that the same system of microsomal hydroxylation is operative within this tissue but also that the hydrocarbon is actually subjected to binding during hydroxylation in the system. There is evidence in fact that the latter condition is attained, for it was found by Conney *et al.* (1957) that mild alkaline hydrolysis (methanolic potassium hydroxide at 3° C. for 24 to 48 hours) was necessary for the quantitative liberation of 3 : 4-benzpyrene after addition to the *in vitro* hydroxylating system. Further investigation is obviously required to establish this point and

other limitations will doubtless be exposed when metabolic studies are extended to other hydrocarbons and other species. The proposals are not put forward in any dogmatic sense, therefore, but as a possible explanation of certain known facts concerning the biochemical hydroxylation of polycyclic hydrocarbons, an explanation which may be checked by direct experimentation.

The final question to be considered is whether these theoretical proposals provide any indication that the process of hydroxylation is associated with the carcinogenic response elicited by certain hydrocarbons. If the non-carcinogenic members of this series do in fact undergo hydroxylation when in an unbound state then an obvious conclusion is that it is the binding, either ortho or para, of the carcinogens which is a contributing factor in carcinogenesis. If, however, the non-carcinogens are first subjected to binding prior to hydroxylation then, as theoretical considerations suggest that this occurs at the reactive bond rather than across the reactive positions, this would appear to exclude the ortho form of binding from the carcinogenic mechanism. Furthermore, the proposal that species differences in metabolism between the rabbit and rat and mouse respectively may be attributed to the greater contribution of ortho binding in the former species is inversely paralleled by the susceptibility of these species towards the induction of carcinogenesis by the hydrocarbons. 1 : 2 : 5 : 6-Dibenzanthracene, for example, is a potent carcinogen for the rat and mouse but is without activity in the rabbit. If the theory is correct, therefore, there would appear to be no association between the ortho or K-region binding of hydroxylation and carcinogenesis. It is tempting to speculate then that the para form of binding may be an important factor governing the carcinogenic response and certain evidence may be cited in support of this view. Thus the blocking of the para-positions in compounds such as cis-9 : 10-dimethyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene and 9 : 10-dimethyl-1 : 2-benzanthracene-α, β-endo-succine acid is accompanied by a marked reduction in the carcinogenic potencies of the parent hydrocarbons from which these are derived. Such behaviour may be interpreted as an indication that substitution in the meso-positions of these hydrocarbons prevents the process of para-binding during hydroxylation. Also, 9 : 10-dimethyl-1 : 2-benzanthracene is considerably more carcinogenic than is the parent 1 : 2-benzanthracene and this behaviour is reflected in the reactivities of these compounds towards the para-addition of maleic anhydride, this occurring more readily with the dimethyl derivative (Bachmann and Chemerda, 1938; Newman and Otsuka, 1959). In other words the effect of methyl substitution in the 9 : 10-positions of 1 : 2-benzanthracene, and indeed in those of anthracene, is to facilitate the process of para-addition at these points. In this case, however, we are dealing with a specific type of cyclic dienophile addition and it is not surprising therefore that the analogy breaks down when applied to the whole range of polycyclic hydrocarbons. What is envisaged biologically rather is an addition type of complex arising from combination of the carcinogen either with two molecules or with distal groups of the same molecule so that it constitutes in effect a cross linkage between the two. As was stated earlier the exact mode of linkage is as yet unknown. Boyland (1950*a*) has suggested a simple covalent type of addition whilst Pullman and Pullman (1955) prefer the quinonoidal type of linkage. A third type of addition not yet considered, however, is that of co-ordination. The latter phenomenon was investigated by Kofahl and Lucas (1954) who reported a fair degree of correlation between the carcinogenic

potencies of aromatic hydrocarbons and their co-ordination activity towards silver ion in the argentation reaction. Complex formation with iodine ions has also been observed (Benesi and Hildebrand, 1948) and the solubilising effect of purine derivatives upon the polycyclic hydrocarbons (Weil-Malherbe, 1946) is now well known. Is it not possible therefore that similar co-ordination complexes may be formed between the hydrocarbon and say the ionised groups of an enzyme within the microsomes? The process of hydroxylation may then be regarded as a neutralisation of the electronic charge resident on the hydrocarbon portion of the complex resulting in the formation of a phenol and consequent liberation of the enzyme.

If, as the evidence suggests, the mechanism of hydroxylation is associated with the carcinogenic mechanism, then the origin of this cellular malformation must reside within the microsomes. Furthermore, as the hydroxylating activity of the microsomes was found by Conney *et al.* (1957) to remain unchanged after preincubation with ribonuclease, the field of action may be narrowed down still further to the non-ribonucleic acid fraction of these organelles. It is significant therefore that Fiala and Fiala (1959) have concluded on entirely different grounds that the non-ribonucleoprotein fraction of the ergastoplasm is the origin of the hepatic carcinogenic response elicited by azo dyes.

The suggestion, however, that there may be an association between a para form of binding and carcinogenesis is in conflict with certain experimental evidence on this subject.

The hydrocarbon 1 : 2 : 3 : 4-dibenzanthracene for example, does not possess a K-region as such and yet undergoes extensive binding to protein within the skin (Heidelberger and Moldenhauer, 1956). The logical explanation of this behaviour is that binding occurs at the reactive meso-positions of the molecule and yet this hydrocarbon is, at most, a very weak carcinogen. Also, the evidence reported by Oliverio and Heidelberger (1958) in support of a relationship between K-region binding and carcinogenic activity in the 1 : 2 : 5 : 6-dibenzanthracene series is most convincing although not without certain anomalies.

What is possible, however, is that we are dealing with two different and possibly independent processes, one concerned with overall binding to cellular protein and the other with binding, possibly to enzymes, within the microsomes where the hydroxylating system is active. There is in fact evidence that two such forms of binding may exist, for it was found by Miller (1951) that, although normal alkaline hydrolysis was effective in liberating a fluorescent acidic derivative from the precipitated protein of skin following treatment with 3 : 4-benzpyrene, a further fluorescent neutral fraction was only released by this procedure when zinc dust was present in the mixture. Significantly the former conditions are also those necessary for the quantitative liberation of 3 : 4-benzpyrene from the microsomal system of hydroxylation (Conney *et al.*, 1957).

On existing evidence the K-region binding theory of carcinogenesis must obviously be favoured for this phenomenon has been demonstrated within the skin where tumour formation occurs. The microsomal system of hydroxylation on the other hand has so far been detected only within the liver where the induction of hepatoma formation by polycyclic aromatic hydrocarbons is open to doubt. More must be known therefore of the enzymatic system of hydroxylation within the skin and the mechanisms involved before this apparent conflict can be resolved. It is then possible that studies of binding in the *in vitro* systems may

provide a truer picture of *in vivo* behaviour for, as the hydrocarbon is not added until the processes of cellular disintegration and centrifugation have been carried out, the objections raised by Hadler, Darchun and Lee (1959) no longer apply.

Except where stated the chemical data referred to in this work are taken either from Fieser and Fieser (1944), Gilman's ' Organic Chemistry ' (1947) or Elsevier's ' Encyclopaedia of Organic Chemistry ', Supplementary Volume 14 (1951). Data of carcinogenic activity are taken from Hartwell (1951).

SUMMARY

A theory involving linkage to cellular material, possibly enzymes, within the microsomes has been developed to account for the fact that carcinogenic hydrocarbons undergo hydroxylation principally at positions of the molecule which are inert to chemical attack. It is postulated that binding may occur either across non-adjacent reactive positions or at the reactive bond each of which leads to activation at different sites of the molecule. On this reasoning it is concluded that species differences in the site of hydroxylation are due to differences in the relative contributions of these distinct forms of binding to the total amount bound in any one species. An ortho form of binding would appear to be favoured in the rabbit and a para form in the rat and mouse.

The hydroxylation of non-carcinogenic hydrocarbons on the other hand is best explained either on the basis of non-binding or of binding through the most reactive bond. There would thus appear to be no association between an ortho form of binding during hydroxylation and carcinogenesis. It follows therefore that a para form of binding may be of importance in this respect and this possibility is discussed in relation to current views on protein binding and carcinogenesis.

The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

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METABOLISM OF 1 : 2-BENZANTHRACENE IN THE RABBIT

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Received for publication October 31, 1959

THE metabolism of 1 : 2-benzanthracene in rats and mice was investigated by Berenblum and Schoental (1943) who reported the excretion in the faeces of a phenolic derivative identified as 4′-hydroxybenzanthracene by methylation and spectroscopic comparison with known methoxybenzanthracenes. Recent studies by the author (Harper, 1959*a*) confirmed this identification for the mouse faecal metabolite but the spectral analyses were considered to be consistent with the presence of an additional phenolic derivative. The latter compound did in fact appear to be the major component of the initial phenolic fraction conjugated with sulphuric acid in the bile. It was suggested, by analogy with other hydrocarbons, that this may be the 2′-hydroxy derivative and theoretical considerations (Harper, 1959*b*) led to the proposal that the formation of this metabolite would be favoured in the rabbit. Samples of both 2′-hydroxy- and 2′-methoxybenzanthracene have now been made available through the kind generosities of Professor Badger in Australia and Dr. Schoental in this country so that it has been possible to test these proposals by experiment.

MATERIALS AND METHODS

The two rabbits used in this work were maintained on a green-free diet for two weeks prior to injection in order to reduce the chlorophyll content of the gut and faeces.

Purified 1 : 2-benzanthracene was prepared for injection either as a colloid in distilled water (1 mg. per c.c.) or as a supersaturated solution in warm tricaprylin (20 mg. per c.c.).

In the first experiment (Experiment A) one rabbit was injected intraperitoneally with 10 c.c. of the tricaprylin solution and the faeces were collected daily for a period of one week. Extraction into xylene via acetone was carried out immediately and the phenolic metabolite was isolated from the pooled xylenes as described previously (Harper, 1959*a*).

In the second experiment (Experiment B) the remaining rabbit was injected intravenously via the ear vein with 20 c.c. of colloidal hydrocarbon and killed at 2 hours. No fluorescence attributable to the hydrocarbon or its metabolites was discernible within the tissues and the combined gall bladder and small intestine were therefore homogenised and subjected to hot acid hydrolysis (1 N hydrochloric acid at 100° C. for 1 hour under nitrogen). Extraction with xylene then removed a blue-fluorescent derivative which was isolated by the procedure referred to above for the faecal metabolite.

RESULTS

Experiment A

A solution of the metabolite in ethanol possessed a bright blue fluorescence which changed immediately to yellow on addition of sodium hydroxide. Absorption spectroscopy in hexane revealed the presence of absorbing impurity in the shorter wave length region but above 280 mμ the bands recorded (Fig. 1, Table I) were, with one exception (see DISCUSSION), almost identical with those of synthetic 2′-hydroxybenzanthracene.

TABLE I

Absorption bands in hexane (mμ)

Rabbit faecal metabolite	2′-OH	Mouse metabolite BA-OH	Methylated rabbit metabolite	2′-OMe
—	226–227	226	226–227	227
—	[255–256]	253	[254–256]	[254–256]
—	262	[262]	262–263	262–263
—	269–270	[270–272]	270	269
—	[278–280]	[278–280]	278–280	280
286	286	286	287	287
297	297	297	297	297·5
322–324	325·5	[320–324]	324–326	326–327
337	—	337	—	—
—	341	[340–341]	338–342	340–342
354	354	354–355	354	354·5
373	373	372	372	372
394	394	394	393	393

2′-OH = 2′-hydroxybenzanthracene.
2′-OMe = 2′-methoxybenzanthracene.
BA-OH = free phenolic component of the mouse sulphate conjugate.

Methylation of the metabolite with dimethyl sulphate and excess sodium hydroxide yielded a neutral derivative, purified by chromatography on alumina from benzene followed by alumina from cyclohexane. The absorption spectrum of this compound proved to be almost identical with that of 2′-methoxybenzanthracene (Fig. 2, Table I) and the phenolic derivative excreted in the faeces was therefore concluded to be 2′-hydroxybenzanthracene.

Also included for comparison in the above data (Fig. 1, Table I) is the absorption spectrum of the phenolic fraction obtained from the sulphate conjugate of mouse bile (Harper, 1959*a*). This again is very similar to that of the 2′-hydroxy derivative but differs from it in the sharpness of the major band at 286 mμ. In this respect it is similar to 4′-hydroxybenzanthracene and a probable explanation of this behaviour is that a mixture of the 2′- and 4′-hydroxy compounds is present, with the former in excess.

Experiment B

The primary aim of this experiment, to isolate conjugated metabolites from the gall bladder and small intestine, was prevented by the large amount of acidic colouring matter present in these organs. Recourse was had therefore to the hydrolytic procedure described under MATERIALS AND METHODS in the hope

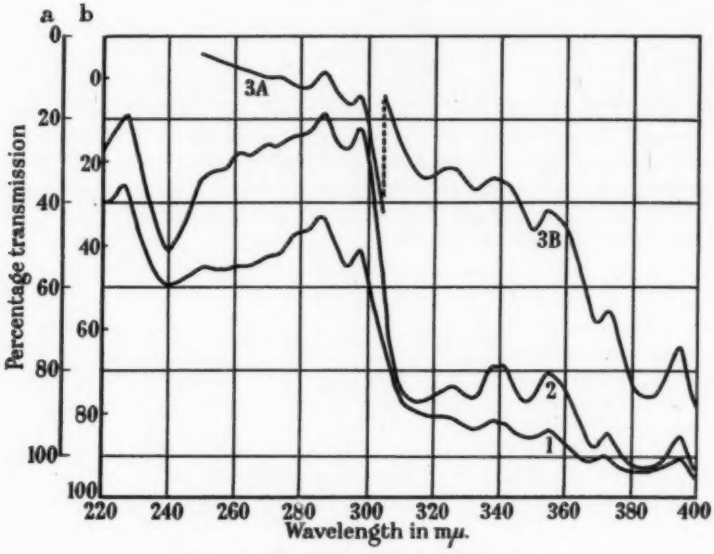

FIG. 1.—Absorption spectra in hexane.

1. Phenolic component of the mouse sulphate conjugate (*b* ordinates).
2. 2′-Hydroxy-1 : 2-benzanthracene (*b* ordinates).

3A and 3B.—Weak and concentrated solutions of the phenolic metabolite of benzanthracene from rabbit faeces (3A—*a* ordinates ; 3B—*b* ordinates).

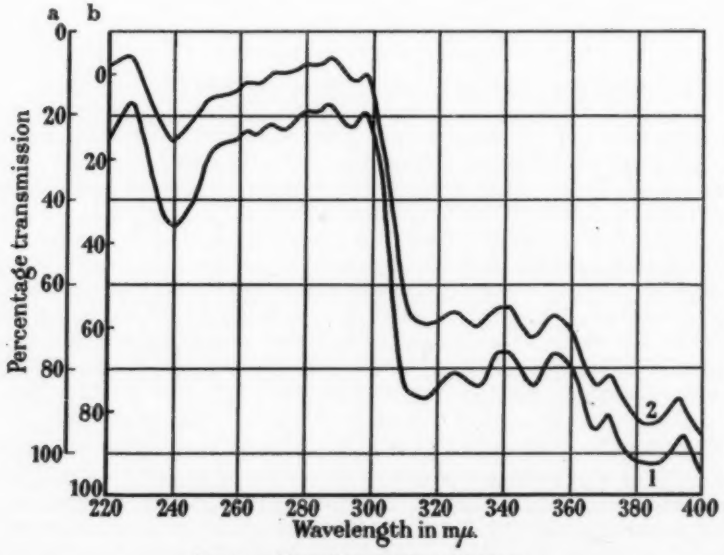

FIG. 2.—Absorption spectra in hexane.

1. 2′-Methoxy-1 : 2-benzanthracene (*b* ordinates).
2. Methylated phenolic metabolite of benzanthracene from rabbit faeces (*a* ordinates).

that a phenolic derivative liberated by this means from a sulphate conjugate would be more amenable to isolation and purification. In this respect the experiment was a success and the phenolic fraction thus obtained, although impure, possessed absorption bands identical with those of the faecal metabolite.

DISCUSSION

Although there can be little doubt that the rabbit metabolite isolated in this work is 2′-hydroxy-benzanthracene it is perhaps surprising, in view of the close correspondence existing between other bands, that the rather diffuse 341 mμ band of the synthetic compound should appear at 337 mμ in the metabolite. This divergence, however, is undoubtedly due to the presence of absorbing impurity for, as absorption in a mixture is additive, the superimposed effect of decreasing background absorption upon a broad, almost horizontal maximum of this nature will be to decrease absorption in the band passing from shorter to longer wave lengths. The overall effect therefore will be a sharpening and hypsochromic displacement of the maximum. Significantly, this effect was not observed with the purer methylated form of the metabolite.

The isolation of free 2′-hydroxybenzanthracene from the gall bladder and small intestine following acid hydrolysis is consistent with the presence in these organs of a sulphate conjugate, if not perhaps the glucuronide, of the phenol. The situation in respect therefore is probably similar to that occurring in the mouse but differs from it in the fact that hydroxylation occurs exclusively in the 2′-position. The metabolism of 1 : 2-benzanthracene in the mouse is indeed analogous to that of 3 : 4-benzpyrene for, although hydroxylation occurs at more than one point of the molecule, the quantitative relationship existing between the resultant products appears to be time dependent. Thus the initial major products yielded by these two hydrocarbons *in vivo* are probably 2′-hydroxy-benzanthracene and the unidentified F_1 metabolite of 3 : 4-benzpyrene but at later time intervals these are replaced by the structurally analogous 4′-hydroxy-benzanthracene and 8-hydroxybenzpyrene. It would appear therefore that the initial products of hydroxylation in the mouse are themselves inhibitors of their own synthesis and herein may lie the secret both of species differences in the site of hydroxylation and in the graded carcinogenic response elicited by the hydrocarbons in these species.

In conclusion it can be said that the hydroxylation of 1 : 2-benzanthracene in the 2′-position and the lack of carcinogenic activity in the rabbit is consistent with the proposal advanced previously (Harper, 1959*b*) that an ortho form of binding is favoured during hydroxylation in the microsomes of this species. The carcinogenic action of this hydrocarbon in the mouse is then to be envisaged as a secondary effect arising from an initial "poisoning" of the defensive ortho-mechanism which then allows the active para form to come into play. Similar considerations may also apply in the case of 1 : 2 : 5 : 6-dibenzanthracene (without activity in the rabbit) and 3 : 4-benzpyrene (reduced activity in the rabbit).

Data of carcinogenic activity are taken from Hartwell (1951).

SUMMARY

1. 1 : 2-Benzanthracene has been found to undergo hydroxylation in the 2′-position after intraperitoneal and intravenous injection in the rabbit. The

phenolic metabolite, 2′-hydroxybenzanthracene, is excreted in the faeces although it is probably eliminated initially via the bile in conjugated form.

2. Previous data obtained on the metabolism of 1 : 2-benzanthracene in mice have been re-examined and are consistent with the view that hydroxylation occurs in both the 2′- and 4′-positions with the former predominating during the initial stages of metabolism.

3. These results are discussed in relation to former proposals concerning hydroxylation and carcinogenesis.

I am greatly indebted to Dr. R. Schoental for her gift of 2′-methoxybenzanthracene and to Professor G. M. Badger for his gift of 2′- hydroxybenzanthracene.

The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

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CONCERNING THE ORIGIN OF SUBSTITUENTS IN POLAR-BOUND DERIVATIVES OF 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE

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Received for publication October 23, 1959

FOLLOWING the demonstration by Miller and Miller (1947) of the binding of carcinogenic aminoazo dyes to proteins of the target organ, much work has been directed towards elucidating the reactive sites of both carcinogen and protein.

Miller and Miller (1953) have suggested that the linkage between dye and protein is stable to treatment with hot ethanolic KOH. Under these conditions hydrolysis of protein occurs so that the recovered polar dye should have an amino acid residue attached. To date, insufficient quantities of this polar derivative have been obtained to enable chemical identification of this residue.

Gelboin, Miller and Miller (1958) have reported that, a few hours after the intraperitoneal administration of a large dose of 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB), protein-bound derivatives are detectable in the liver. The electrophoretic and spectral properties of the bound dyes obtained, either by prolonged feeding or following a single dose, were similar. The findings of Gelboin, Miller and Miller (1958) support the view that a large percentage of the bound dye is bound to certain liver proteins during their synthesis rather than by becoming attached to protein already formed. This interpretation is in disagreement with that of Hultin (1956) who suggested that the formation of protein-bound dye is independent of protein synthesis.

To date, little evidence is available concerning the actual site(s) involved in the protein(s) concerned. Kusama and Terayama (1957), Terayama and Kusama (1957), Kusama, Terayama and Teruya (1958) and Terayama, Kusama, Teruya, Kuroda and Nakayama (1958), as a result of spectroscopic studies of synthetic polar-like dyes, have suggested that a tyrosine-aminoazobenzene combination is the most probable model for the polar dye.

In this communication the results of an investigation, using isotopically labelled amino acids in an endeavour to determine the origin of substituents in the polar derivative(s) of aminoazo dyes, are presented.

METHODS

Care of animals

As a locally available strain of Wistar rats had previously been found to give higher levels of protein-bound dye than rats of Sprague-Dawley strain, the former were used.

Three of four rats, weighing 18–31 g. were removed from their mother and kept warm for five hours. They were then injected intraperitoneally with 0·5 ml. of a

2 per cent solution of 3'MeDAB in olive oil and returned to the mother after the remaining members of the litter had been removed. After a further hour the young rats were inspected and ones selected were injected intraperitoneally with ^{14}C labelled amino acid.* A dose of 0·01 mc of amino acid was given when the L isomer was used while 0·020–0·025 mc was given when DL amino acids were employed. The following amino acids were used: DL Alanine-1-^{14}C, L Arginine-^{14}C (G), L Aspartic acid-^{14}C (G), L Glutamic acid-^{14}C (G), Glycine-1-^{14}C, L Histidine-2(ring)-^{14}C, *Iso*-Leucine-^{14}C (G), DL Leucine-1-^{14}C, L Lysine-^{14}C (G), L Phenylalanine-^{14}C (G), L Proline-^{14}C (G), L Serine-3-^{14}C, L Serine-^{14}C (G), L Threonine-^{14}C (G), DL Tryptophan (Indolyl-Alanine-3-^{14}C), L Tyrosine-^{14}C (G), DL Valine-1-^{14}C, DL Cystine-^{35}S and DL Methionine-^{35}S. After a further period of 19 hours the rats were sacrificed and their livers removed and homogenized in 2 ml. of water.

Preparation of polar dyes, chromatography and counting techniques

After adding an aliquot of 0·5 M pH 4·0 acetate buffer the liver proteins were precipitated by boiling for three minutes. The precipitated proteins were collected on hardened filter paper, washed with ethanol and then extracted for 48 hours with ethanol in a Soxhlet apparatus. The extracted powders were then digested in ethanolic KOH at 80° C. for 20 hours and the digests extracted twice with 1 : 5 ethanol-ethyl ether by the method of Miller and Miller (1947). The combined ethereal extracts were transferred to small beakers. KOH dissolved in the ethereal extracts was precipitated as carbonate by the addition of a small piece of dry ice and the extract was evaporated to dryness, initially over a water bath and finally *in vacuo*. The residues (apart from potassium carbonate) were transferred by dissolving in 1 : 5 ethanol-ethyl ether to 2·2 cm. diameter watch glasses and any activity determined by using a Geiger Muller tube with a mica window 2·3 mg. cm.$^{-2}$; counting was performed at infinite thinness for a minimum time of 30 minutes for each sample (background 7–10 counts per minute).

The samples were transferred to Whatman No. 1 paper with a small amount of ethanol. The total amount of polar dye extracted from one liver was introduced on to the paper in three spots. If necessary additional unlabelled polar dye eluted from other chromatograms was added to make the spots visible. The spots were dried with a hair drier between each application and then chromatographed using the solvent system *n*-butanol, acetic acid, water 4 : 1 : 5. Prior to chromatography the spots were exposed to ammonia vapour. After chromatography and drying, the papers were exposed to HCl vapour and any areas showing a faint pink colour were cut out and eluted with ethanol in a semi-micro Soxhlet apparatus. After standing under a hood for 3–4 hours the remainder of the paper was sprayed with ninhydrin. After drying *in vacuo* the eluted material from the pink spots was then rechromatographed on Whatman No. 1 paper using the system benzyl alcohol, water and once again the areas showing a faint pink colour on acidification were cut out and eluted with ethanol. All the polar dye arising from a single liver (which was run in three spots on the initial chromatograms) was then pooled and run as a single spot in the benzyl alcohol, water system. After drying, the diluted material was transferred to watch glasses and counted as before at infinite thinness, for a minimum time of 30 minutes. The density of material on the sources counted was not greater than 10 μg. cm.$^{-2}$ The material counted in each case was

* Obtained from the Radiochemical Centre, Amersham, Bucks., England.

thus the total amount of polar dye derived from a single liver. Counting was performed for at least half an hour with each source ; background varied between 8 and 10 counts per minute. The amount of azo dye present in each source was estimated spectrophotometrically in HCl-ethanol at 520 mμ. A solution of 3′MeDAB in acid-ethanol was used as a standard.

In all cases control spots of known unlabelled amino acid were run on the same piece of paper with the dye-labelled amino acid samples. As a result of a preliminary screening experiment tryptophan, valine, histidine and proline were subjected to further study. Using these amino acids, methionine and cystine, litter mates were given either a labelled amino acid alone or a labelled amino acid plus carcinogen and their livers subsequently hydrolyzed and extracted in an identical manner. Both samples were then chromatographed on the same sheet of paper and corresponding areas were cut out, eluted and counted.

All chromatography was performed at 23 ± 2° C.

RESULTS

The polar derivatives of 3′MeDAB prepared from livers of rats given ^{14}C labelled alanine arginine, aspartic acid, glutamic acid, glycine, histidine, *iso*-leucine, leucine, lysine, phenylalanine, proline, serine, threonine and tyrosine showed little or no activity. All of these experiments were run at least in duplicate ; in some cases the procedure was carried out four times.

The polar dyes obtained from rats given labelled tryptophan, valine, methionine and cystine showed appreciable activity (Table I). While material prepared

TABLE I.—*Showing Activities of Polar Dyes Obtained from Rats given Labelled Tryptophan, Valine, Cystine and Methionine*

Amino acid	Number of rats	Mean counts per minute per mg. DF polar dye	Level of statistical significance of difference between group given carcinogen and control group (*t*-test)
Tryptophan	8	4495 ± 285*	<1%
Valine	7	3268 ± 252*	<1%
Cystine	4	2887 ± 356*	6%
Methionine	4	4420 ± 962	Not significant

* = Standard deviation.

in the same way from rats given labelled tryptophan only (without 3′MeDAB) or labelled valine only showed very low activity, that prepared from rats given ^{35}S labelled methionine or cystine only showed some activity.

Rf values of amino acids and aminoazo dyes are shown in Table II.

DISCUSSION

Interpretations drawn from studies of this type must be viewed within the limitations of the technique. The metabolic interconversion of amino acids is well established so that if the polar derivative of 3′MeDAB prepared from rats given a particular ^{14}C labelled amino acid shows a significant but low activity, this should

TABLE II.—*Showing Rf Values of Amino Acids and Azo Dyes in Solvent Systems Used*

Substance	Benzyl alcohol-water	*n*-Butanol, acetic acid water—4 : 1 : 5
Alanine	0·04	0·39
Arginine	0·01	0·19
Aspartic acid	0·00	0·33
Glutamic acid	0·00	0·37
Glycine	0·02	0·33
Histidine	0·02	0·19
Iso-Leucine	0·18	0·70
Leucine	0·20	0·72
Lysine	0·00	0·18
Phenylalanine	0·37	0·66
Proline	0·12	0·43
Serine	0·01	0·31
Threonine	0·02	—
Tryptophan	0·29	0·61
Tyrosine	0·14	0·53
Valine	0·11	0·56
Cystine	0·00	—
Methionine	0·17	0·57
Polar dye*	0·80	0·75
3′MeDAB	0·94	0·94
3′MeMAB	1·00	0·93
3′MeAB†	1·00	0·94

* The dye recovered after hydrolysis of the liver proteins has polar properties as demonstrated by Miller and Miller (1947) and was called by them polar dye. Its exact structure has not been determined as yet.

† Presented by Dr. J. A. Miller.

be considered as being due to the amino acid given being converted to another amino acid which becomes bound. On the other hand if the polar dye obtained, after giving a particular labelled amino acid, shows high activity this might possibly indicate that this amino acid marks the site of attachment of dye to protein. In order to reduce this interconversion of amino acids the time taken from administration of the dye and labelled amino acids should be reduced to a minimum. The observation of Gelboin, Miller and Miller (1958) that the polar dye formed within hours of giving a large dose of 3′MeDAB is similar to that formed on prolonged feeding of lower levels of dye, offers an opportunity of greatly reducing this time but still retaining a high level of bound dye. Thus the time of 19 hours was determined by the interval required to achieve sufficient dye binding to enable recognition of the dye on the chromatograms. This is considerably longer than the time required for maximal labelling of proteins by giving amino acids.

Infant rats were used in order to get the maximum concentration of labelling of polar dye with associated economy of isotopes. Since Gelboin, Miller and Miller have correlated the rapid achievement of a high level of bound dye with the rate of protein synthesis the rats used were fasted but returned to the mother shortly before injection.

Preliminary investigations were made concerning the toxicity of large amounts of certain unlabelled amino acids to infant rats in an endeavour to investigate the possibility of decreasing the conversion of one amino acid into another in subsequent experiments using isotopes; for example, in view of the conversion

of serine to cystine it was considered that the administration of a large excess of unlabelled cystine with labelled serine would decrease the formation of labelled cystine from the serine. However, as the combined injections of 3′MeDAB and a large excess of an amino acid frequently was found to be toxic to suckling rats, this procedure was not adopted in the experiments using isotopically labelled amino acids.

In all experiments employing isotopic techniques most stringent purification methods are essential. The extracted dyes were purified by chromatography using the systems *n*-butanol, acetic acid, water 4 : 1 : 5 and benzyl alcohol-water. Control spots of free amino acids were run on the same sheets of paper and these showed a clear separation between the polar dye and the free amino acids. The Rf values of the amino acids and aminoazo dyes are sufficiently different, in the solvent system used, to obtain a good separation (Table II). In each case a ninhydrin reaction carried out after cutting out the polar dye spots showed no ninhydrin positive material at the margin of the spot.

Before attributing activity to the presence of any amino acid as a substituent in the polar dye molecule it is necessary to demonstrate that the material, prepared in the same way from the livers of rats given labelled amino acid but no carcinogen, shows little or no activity. This excludes the possibility that the activity observed is due to any small peptide or other metabolite of the amino acid in question with Rf values similar to that of the polar bound dye.

Appreciable activity was obtained only with the polar dyes derived from rats given labelled tryptophan, valine, cystine or methionine. In the cases of cystine and methionine, however, some activity was also found in corresponding material prepared from rats given labelled amino acid only.

Application of the *t*-test to the results (group given 3′MeDAB plus labelled amino acid and group given labelled amino acid alone) gave values which were significant at the 1 per cent level for tryptophan and valine and at the 6 per cent level for cystine. No statistically significant difference between the methionine groups could be detected.

The results obtained suggest that tryptophan, valine and possibly cystine (or cysteine) residues are associated with the polar bound dye.

Since in these experiments no activity was found in the polar dye recovered from rats given dye and either labelled tyrosine or its precursor phenylalanine, it is difficult to reconcile these results with the suggestion of Kusama and Terayama (1957), Terayama and Kusama (1957), Kusama *et al.* (1958) and Terayama *et al.* (1958), that a tyrosine residue is involved.

It is appreciated that these results are based on experiments on few animals. Further work is contemplated.

SUMMARY

1. Suckling rats have been given 3′MeDAB and individual labelled amino acids. The recovered polar dyes have been purified by chromatography and examined for activity.

2. Activity has been found in the polar dyes prepared from rats given labelled tryptophan, valine, cystine and methionine.

3. It is suggested that tryptophan, valine and possibly cystine (cysteine) residues are the substituents in the polar derivatives.

This work was supported by a grant from the Anti-Cancer Council of Victoria. Statistical advice from Miss B. Laby, Department of Statistics, University of Melbourne, is gratefully acknowledged.

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INDIRECT EFFECT OF X-RADIATION ON THE RESPIRATORY METABOLISM OF EHRLICH ASCITES TUMOUR CELLS AND MITOCHONDRIA

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Received for publication October 9, 1959

It has been found previously (Silk, Hawtrey and Macintosh, 1958) that Ehrlich ascites cells show lower oxygen uptake and aerobic glycolysis in a medium of peripheral lymph drained from X-irradiated dog tissues than in a medium of normal peripheral dog lymph. Since the ascites cells themselves were not irradiated, an " indirect " action of radiation was postulated to account for the observed effects on respiratory metabolism which operate through the environmental medium.

In a further examination of this " indirect " action of radiation, the respiratory metabolism of intact Ehrlich ascites cells and of Ehrlich ascites cell mitochondria has been compared in media previously incubated with mouse antero-lateral abdominal wall and in the same media given 1000 roentgen X-irradiation in contact with the tissue before incubation. Although the glycolysis of intact cells is little different in the non-irradiated and X-irradiated media, the oxygen uptake of both intact cells and mitochondria is markedly influenced. Measurements of respiration have been made in both bicarbonate and phosphate buffered media. As in previous work (Silk, Hawtrey and Macintosh, 1958) the effects are found to be opposite in the two buffer systems.

The reasons for this " indirect " effect of radiation has been sought in the chemical composition of the environmental media before and after radiation. It would appear that factors liberated by the irradiated mouse antero-lateral abdominal wall are capable of influencing the respiration rate of Ehrlich ascites cells and mitochondria.

MATERIALS AND METHODS

Mouse antero-lateral abdominal wall

Mice were killed by cervical fracture. A median incision was then made in the ventral aspect of the abdomen. Flaps of skin and subcutaneous tissue were dissected away up to the dorsal aspect of the abdominal cavity. The remainder of the antero-lateral abdominal wall consisting of peritoneum, subserous tissue and fascia with layers of muscle and overlying connective tissue, was then excised for experiment. The tissue was cut into portions approximately 1 cm. square,

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and stored in ice cold normal saline +0·2 per cent w/v glucose until ready for incubation with various media.

Preparation of media for incubation with mouse antero-lateral abdominal wall

(a) *Ehrlich ascites plasma.*—Citrated (5 mg./ml.) Ehrlich ascites fluid was centrifuged at 4° C., to give clear ascites plasma free of cells. The bicarbonate content of an aliquot was determined by acidification in Warburg manometers filled with 95 per cent O_2 + 5 per cent CO_2, and the calculated amount of 0·75 M $NaHCO_3$ solution was then added to the main bulk to give a final bicarbonate concentration of approximately 0·017 M. The pH values of all samples used lay within the range pH 7·2–7·4 when measured in contact with a gas mixture containing 95 per cent O_2 + 5 per cent CO_2, and agreed generally to within 0·2 of a pH unit for corresponding batches of non-irradiated and 1000 roentgen X-irradiated media. For use as a medium in which to determine both the glycolysis and respiration of intact Ehrlich ascites cells, the plasma was fortified with 0·25 per cent w/v glucose before incubation with mouse tissue. For use as a medium in which to determine only the O_2 uptake of intact cells, 1·30 ml. of 0·3 M potassium succinate was added to each 60 ml. in place of the glucose. This amount of succinate was adequate to serve as substrate for the mouse antero-lateral abdominal wall tissue during incubation, and also for respiration of the Ehrlich cells allowed to metabolise in the medium after incubation.

(b) *Ringer bicarbonate-succinate.*—The medium contained 1·30 ml. of 0·3 M potassium succinate per 60 ml. of standard Ringer bicarbonate solution.

(c) *Ringer phosphate-succinate.*—The medium contained 1·30 ml. of 0·3 M potassium succinate per 60 ml. of standard Ringer phosphate solution.

(d) *Phosphate buffered medium.*—The following medium was prepared: phosphate buffer (pH 7·4), 0·067 M; $MgCl_2$, 0·009 M; KCl, 0·015 M; glucose, 0·015 M; sucrose, 0·044 M.

Radiation and incubation with mouse antero-lateral abdominal wall

Precautions were taken to ensure sterility during all operations.

Aliquots (30 ml.) of the required medium (*a*), (*b*), (*c*) or (*d*), were placed in pairs of 250 ml. conical flasks. Antero-lateral abdominal wall sections from 6 mice (approximately 2·4 g. wet wt.) were placed in each of the flasks. One flask of each pair was then given 1000 roentgen X-irradiation (220 kV, 15 mA, filtered through 0·2 mm. Sn + 0·25 mm. Cu + 1·0 mm. Al) at the rate of 160 roentgen/min. delivered through the base of the vessel.

Both the irradiated and non-irradiated flasks of each pair were then incubated 3 hours with slow shaking on a Warburg bath at 37° C. During incubation of bicarbonate media 200 c.c./min. of water-saturated 95 per cent O_2 + 5 per cent CO_2 gas mixture was blown through the flasks. Suspensions in phosphate buffered medium were similarly gassed with 200 c.c./min. pure O_2.

After incubation the media were freed of all cells by centrifugation at 2500 revs/min. for 15–20 minutes.

Ascites plasma (*a*).—The bicarbonate concentration was re-adjusted to 0·017 M as described above. For use as a medium in which to determine glycolysis, extra glucose was added in amount equivalent to the bicarbonate lost through fermentative lactic acid production during incubation with the tissue.

Ringer bicarbonate-succinate (*b*).—The bicarbonate concentration was adjusted to 0·195 M after incubation. No extra succinate was added.

Ringer phosphate-succinate (*c*) *and Phosphate buffered medium* (*d*) were carefully triturated to pH 7·5 with KOH after incubation and were used without further addition of succinate or glucose.

All media were filtered through sintered glass (Porosity 5) and were stored in the cold before use.

Deproteinisation of Ehrlich ascites plasma after incubation with mouse antero-lateral abdominal wall

Immediately after incubation the plasma was deproteinised by addition of 4 vols. cold acid-free ethanol. After standing overnight at 4° C. the precipitate was removed by centrifugation. Alcohol was removed from the supernatant by concentration under reduced temperature and pressure to ¼ of the original plasma volume. The solution was then made up to the original plasma volume by addition of distilled water. The bicarbonate concentration was re-adjusted to 0·017 M as above, and the medium passed through a sintered glass (Porosity 5) filter before use.

Ehrlich mouse ascites carcinoma cells

The tumour was kindly supplied by Dr. Kanematsu Sugiura of the Sloan-Kettering Institute, N.Y., and has been maintained in Swiss albino mice which were used between the 9th and 12th day after inoculation.

The total cells present in a chilled 10 ml. sample of citrated (5 mg./ml.) ascites fluid was removed by centrifugation at 4° C. For experiments with bicarbonate buffered media the cells were washed by re-suspending in 2–3 vols. Ringer bicarbonate-succinate solution (*b*). For experiments with phosphate buffered media the cells were washed in the appropriate solution (*c*) or (*d*). Weighed portions of each washed cell mass were then thoroughly slurried with aliquots of corresponding non-irradiated and X-irradiated media (150 mg./4·5 ml. for bicarbonate buffered media or 420 mg./7·0 ml. for phosphate buffered media). Aliquots (2·0 ml.) of the resulting suspensions were used for manometric measurement of $Q_G^{O_2}$ and Q_{O_2}.

The following abbreviations are used in the text:

$Q_G^{O_2}$	= Aerobic glycolysis in terms of μl. CO_2 per mg. dry weight of tissue per hour at 37° C.
Q_{O_2} (intact cells)	= μl. O_2 uptake per mg. dry weight of cells per hour at 37° C.
Q_{O_2} (mitochondria)	= μl. O_2 uptake per mg. protein per hour at 30° C.
ADP	= adenosine diphosphate.
AMP	= adenosine monophosphate.
DPN	= diphosphopyridine nucleotide.
EDTA	= ethylene diamine tetra-acetic acid.
TCA	= trichloro-acetic acid.

Bicarbonate containing media were equilibrated with 95 per cent O_2 + 5 per cent CO_2 in a small pressure bomb at 30 p.s.i. before use. Phosphate buffered media were equilibrated with pure O_2.

Ehrlich ascites cell mitochondria

These particulates were isolated in 0·25 M sucrose according to the method of Hawtrey and Silk (1960). Saline washed Ehrlich ascites cells were previously

swollen 15 minutes in hypotonic Ringer phosphate solution and then lysed into pure water using a Dounce homogeniser for 3·0 minutes. Immediately after 3 minutes, concentrated sucrose solution was added to give a 0·25 M sucrose homogenate from which the mitochondria were isolated by differential centrifugation in the customary manner. Twice washed mitochondria free of "fluffy layer" were used for all experiments. Each 1·0 ml. of the final suspension in 0·25 M sucrose contained 3–11 mg. of mitochondrial protein determined according to Cleland and Slater (1953).

Aerobic glycolysis and oxygen uptake of intact Ehrlich ascites cells in bicarbonate buffered media

Aerobic glycolysis and oxygen uptake were determined simultaneously by the method of Dixon and Keilin (1933) using Barcroft differential manometers. The precautions described by Elliott and Schroeder (1934) and Dixon (1951) were observed. Measurements were made at 37° C. and the flasks were gassed with water-saturated 95 per cent O_2 + 5 per cent CO_2 during a 15 minute equilibration period. The side arms contained 0·5 ml. of 1·0 M HCl and the stopper cavities were filled with 40 per cent KOH. Clerici solution ($P_0 = 2500$) was used in the manometers.

The two flasks of each manometer contained identical aliquots of either (*a*) the Ehrlich ascites cell suspension in medium incubated with tissue but not irradiated, or (*b*) the Ehrlich ascites cell suspension in medium incubated with mouse abdominal wall tissue after 1000 roentgen X-irradiation.

Experiments were conducted over periods of 40–50 minutes during which time the rates of glycolysis and respiration remained apparently undiminished.

The pH at the beginning and end of each experiment was calculated using the Henderson-Hassalbalch equation with the values $pK^1 = 6{\cdot}1$, $\alpha = 0{\cdot}51$ (c.c. CO_2 dissolved per ml. of medium at 37° C. and 760 mm.) for ascites serum and $pK^1 = 6{\cdot}32$, $\alpha = 0{\cdot}57$ for non-proteinaceous media. The initial pH values of all suspensions were found to lie within the range pH 7·2–7·4.

Retention curves necessary to correct the observed glycolysis for the amount of CO_2 retained by the medium and not registered on the manometer were determined by the method of Brekke and Dixon (1937). The values of R/Δ pH for the X-irradiated medium were on an average 14 per cent lower than for the corresponding non-irradiated medium.

Oxygen uptake and P/O ratio of Ehrlich ascites cell mitochondria in phosphate buffered media incubated with mouse antero-lateral abdominal wall

Corresponding series of Warburg flasks were set up with (a) 2·0 ml. aliquots of phosphate buffered medium incubated with mouse tissue but not irradiated, and (*b*) 2·0 ml. aliquots of corresponding medium incubated with tissue after 1000 roentgen X-irradiation. To each flask was added 0·26 ml. of the following solution: KF, 0·173 M; $MgCl_2$, 0·023 M; ADP, 0·029 M; AMP, 0·012 M; EDTA, 0·023 M; cytochrome-c, $1{\cdot}13 \times 10^{-4}$ M; DPN, 0·0028 M. The side arms of each flask contained 0·24 ml. of 0·3 M glucose plus 1–2 mg. of crystalline hexokinase, and the centre wells 0·2 ml. of 40 per cent KOH solution with a roll of filter paper.

After cooling the flasks in ice, 0·4 ml. of the mitochondrial suspension in 0·25 M. sucrose was added to each vessel.

Final concentrations in each flask were as follows: potassium phosphate buffer (pH 7·4), 0·045 M; KF, 0·015 M; $MgCl_2$, 0·008 M; ADP, 0·0025 M; AMP, 0·001 M; EDTA, 0·002 M; glucose, 0·034 M; KCl, 0·01 M; sucrose 0·062 M; DPN, $2{\cdot}4 \times 10^{-4}$ M; cytochrome-c, $9{\cdot}8 \times 10^{-6}$ M; hexokinase, 1–2 mg. (plus unknown amounts of lactic acid, amino acids, substrates, etc., resulting from incubation of part of the flask contents with mouse antero-lateral abdominal wall).

Experiments were conducted at 30° C. over periods of 25–35 minutes with air as the gas phase. An equilibration time of 13 minutes on the manometer bath was allowed before closing the taps and adding the side-arm contents. The reaction was stopped by addition of 1·0 ml. of 30 per cent TCA to each flask which was then placed in ice. Phosphate uptake was determined by the method of Fiske and Subbarow (1925) on the cold TCA filtrate.

Amino acid analysis

Samples of corresponding non-irradiated and X-irradiated media were deproteinised by addition of 4 vols. ethanol. After standing overnight at 4° C. and centrifuging, 0·2 ml. aliquots of the supernatant were analysed for total free amino acid and peptide content by the quantitative ninhydrin method of Troll and Cannan (1953) using a standard leucine curve.

Protein analysis

Duplicate aliquots of corresponding non-irradiated and X-irradiated media were analysed for total protein content according to the biuret method of Gornall, Bardawill and David (1949).

Assay of materials

Hexokinase was used in the crystalline form supplied by General Biochemicals Inc., Chagrin Falls, Ohio, and was assayed (*a*) by hexose disappearance according to Somogyi (1952) and (*b*) by measurement of acid labile phosphorus disappearance according to Crane and Sols (1955). Preparations were found to have an activity of approximately 150 units per mg.

Cytochrome-c was used as a 1 per cent solution in isotonic saline as supplied by General Biochemicals Inc. and was found to contain $5{\cdot}87 \times 10^{-7}$ moles per ml. when assayed according to Umbreit, Burris and Stauffer (1957).

AMP and *ADP* were used as supplied by General Biochemicals Inc. and by the H.M. Chemical Co. of Los Angeles, California, respectively.

DPN was obtained from General Biochemicals Inc. and was assayed 85 per cent pure by a modification of the dithionite reduction method of Brodie (1955) using 0·2 M phosphate buffer instead of 0·1 M.

All other substrates and chemicals were of Analytical Reagent grade, and where necessary were neutralised to pH 7·4 with potassium hydroxide before use.

RESULTS

(1) *Aerobic glycolysis of Ehrlich ascites cells in ascites plasma incubated with mouse antero-lateral abdominal wall*

The aerobic glycolysis of Ehrlich ascites cells was compared in 6 corresponding batches of ascites plasma incubated with abdominal wall tissue from healthy mice (*a*) non-irradiated and (*b*) 1000 roentgen X-irradiated before incubation.

The results in Table I show that the glycolysis was on an average 15 per cent lower in the X-irradiated medium.

TABLE I.—*Aerobic Glycolysis of Ehrlich Ascites Cells in Ascites Plasma Incubated with Tissue,* (a) *Not Irradiated and* (b) 1000 *Roentgen X-Irradiated before Incubation*

Batch No.	Glycolysis $Q_G^{O_2}$ Not irradiated	X-irradiated	Percentage difference
1	39·5	36·6	−7·3
	30·2	31·6	+4·6
2	36·6	27·9	−23·8
	41·4	20·2	−51·2
3	23·0	21·5	−5·8
	24·8	22·2	−10·5
4	31·1	24·8	−12·7
	28·6	24·3	−15·0
5	31·8	30·3	−4·7
	38·6	27·5	−28·7
6	31·8	26·1	−17·9
	31·0	27·6	−11·0
Average	32·4±4·4*	26·7±3·5	−(15·3±10·1)

* Average deviation from mean.

(2) *Oxygen uptake of Ehrlich ascites cells in ascites plasma incubated with mouse antero-lateral abdominal wall*

The oxygen uptake of Ehrlich ascites cells was compared in 5 corresponding batches of ascites plasma incubated with abdominal wall tissue from healthy mice (*a*) non-irradiated and (*b*) 1000 roentgen X-irradiated before incubation.

The results in Table II show that the oxygen uptake was on an average 43 per cent higher in the media X-irradiated with tissue before incubation.

TABLE II.—*Oxygen Uptake of Ehrlich Ascites Cells in Ascites Plasma Incubated with Tissue* (a) *Not Irradiated and* (b) 1000 *Roentogen X-irradiated before Incubation*

Batch No.	Oxygen uptake Q_{O_2} Not irradiated	X-irradiated	Percentage difference
1	2·01	4·55	+126·3
	3·03	6·04	+99·3
2	6·57	7·26	+10·5
	4·78	5·77	+20·7
3	6·07	8·10	+33·4
	5·38	7·53	+40·0
4	5·54	6·68	+20.6
	6·70	8·92	+33·1
5	5·84	7·32	+25·4
	6·25	7·63	+22·1
Average	5·22±1·17*	6·98±0·98	+(43·1±27·8)

* Average deviation from mean.

(3) *Aerobic glycolysis of Ehrlich ascites cells in ascites plasma incubated with antero-lateral abdominal wall from tumour-bearing mice*

For these experiments the antero-lateral abdominal wall was removed from mice bearing a 10-day Ehrlich ascites tumour. Any small areas of solid tumour formation were carefully removed and macroscopically the tissue appeared normal. Incubation with ascites plasma was carried out as described for antero-lateral abdominal wall from healthy mice.

The aerobic glycolysis of Ehrlich ascites cells was then compared in 3 batches of ascites plasma incubated with the tissue (*a*) non-irradiated and (*b*) 1000 roentgen X-irradiated before incubation.

The results in Table III show that the glycolysis was on an average very slightly lower in the X-irradiated medium than in the corresponding non-irradiated control.

TABLE III.—*Aerobic Glycolysis of Ehrlich Ascites Cells in Ascites Plasma Incubated with Antero-lateral Abdominal Wall from Tumour-bearing Mice* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No.	Glycolysis $Q_{O_2}^{O_2}$ Not irradiated	X-irradiated	Percentage difference
1	33·2	29·2	−12·0
	38·1	37·1	−2·6
2	32·8	33·5	+2·1
3	35·2	33·8	−4·0
	35·0	35·2	+0·6
Average	34·9±1·5*	33·8±1·9	−(3·3±2·8)

* Average deviation from mean.

(4) *Oxygen uptake of Ehrlich ascites cells in ascites plasma incubated with antero-lateral abdominal wall from tumour-bearing mice*

Antero-lateral abdominal wall was removed from mice bearing a 10-day Ehrlich ascites tumour, and was incubated with ascites plasma as above.

The oxygen uptake of Ehrlich ascites cells was then compared in 2 batches of ascites plasma incubated with the tissue (*a*) non-irradiated and (*b*) 1000 roentgen X-irradiated before incubation.

The results in Table IV show that the oxygen uptake was on an average 50 per cent higher in the medium incubated with tissue after X-irradiation. The magnitude of the effect decreased with storage time of the media before use, indicating lability of the factor(s) responsible.

(5) *Oxygen uptake of Ehrlich ascites cells in ascites plasma deproteinised after incubation with mouse antero-lateral abdominal wall*

Ehrlich ascites plasma fortified with potassium succinate was incubated with healthy mouse antero-lateral abdominal wall (*a*) without radiation and (*b*) with 1000 roentgen X-irradiation before incubation. The media were subsequently deproteinised.

The oxygen uptake of Ehrlich ascites cells was then compared in 4 corresponding batches of medium prepared with and without radiation.

TABLE IV.—*Oxygen Uptake of Ehrlich Ascites Cells in Ascites Plasma Incubated with Antero-lateral Abdominal Wall from Tumour-bearing Mice* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated Before Incubation*

Batch No.	Oxygen uptake Q_{O_2} Not irradiated	Oxygen uptake Q_{O_2} X-irradiated	Percentage difference	Storage time† after incubation (days)
1	3·96	5·58	+41·0	5
2	3·00	7·80	+160·0	3
	4·72	4·91	+4·0	8
	5·39	6·21	+17·0	10
Average	4·27±0·79*	6·13±0·88	+(50·5±9·5)	—

* Average deviation from mean.
† Media were stored at −30° C. before experiment.

The results in Table V show that the oxygen uptake was on an average 32 per cent higher in the medium incubated with tissue after X-irradiation.

TABLE V.—*Oxygen uptake of Ehrlich Ascites Cells in Ascites Plasma Deproteinised After Incubation with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No.	Oxygen uptake Q_{O_2} Not irradiated	Oxygen uptake Q_{O_2} X-irradiated	Percentage difference
1	9·00	10·93	+21·5
2	4·15	5·78	+39·2
	5·15	6·85	+33·0
3	7·05	11·10	+57·5
	7·65	9·90	+29·6
4	9·83	11·82	+20·4
	8·86	10·64	+20·2
Average	7·38±1·66*	9·57±1·86	+(31·6±9·9)

* Average deviation from mean.

(6) *Oxygen uptake of Ehrlich ascites cells in Ringer bicarbonate solution incubated with mouse antero-lateral abdominal wall*

In these experiments Ringer bicarbonate fortified with potassium succinate (solution (*b*)) was incubated with healthy mouse antero-lateral abdominal wall (*a*) without radiation and (*b*) with 1000 roentgen X-irradiation before incubation.

The oxygen uptake of Ehrlich ascites cells was then compared in 4 corresponding batches of medium prepared with and without radiation.

The results in Table VI show that the oxygen uptake was on an average 14 per cent higher in the medium incubated with tissue after X-irradiation.

(7) *Oxygen uptake of Ehrlich ascites cells in ascites plasma deproteinised after* 3 *hours' incubation at* 37° *C. without tissue*

Batches of succinate fortified Ehrlich ascites plasma were divided into two portions, one of which was given 1000 roentgen X-irradiation. Both samples were

TABLE VI.—*Oxygen Uptake of Ehrlich Ascites Cells in Ringer Bicarbonate Solution Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No.	Oxygen uptake Q_{O_2} Not irradiated	X-irradiated	Percentage difference
1	11·89	12·41	+4·4
2	6·92	8·72	+26·1
3	9·33	10·62	+13·8
	9·16	10·02	+9·4
4	6·96	7·90	+13·5
	7·02	8·34	+18·8
Average	8·55±1·58*	9·67±1·35	+(14·3±5·4)

* Average deviation from mean.

incubated 3 hours at 37° C. under O_2/CO_2 gas mixture and subsequently deproteinised.

The oxygen uptake of Ehrlich ascites cells was compared in 4 corresponding batches of the deproteinised fluid prepared with and without radiation.

The results in Table VII show that the oxygen uptake was almost identical in corresponding non-irradiated and X-irradiated media.

TABLE VII.—*Oxygen Uptake of Ehrlich Ascites Cells in Ascites Plasma Deproteinised after 3 hours' Incubation at 37° C. Without Tissue* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No.	Oxygen uptake Q_{O_2} Not irradiated	X-irradiated	Percentage difference
1	9·10	9·72	+6·8
2	8·65	8·72	+0·8
	11·20	11·05	−1·3
3	9·78	9·64	−1·4
	8·95	8·50	−5·0
4	9·30	9·10	−2·1
	7·10	7·05	−0·7
Average	9·15±0·80*	9·11±0·88	−(0·4±2·2)

* Average deviation from mean.

(8) *Oxygen uptake of Ehrlich ascites cells in phosphate buffered media incubated with mouse antero-lateral abdominal wall*

Three distinct series of experiments were carried out using phosphate buffered media.

(1) Phosphate buffered medium containing excess glucose (solution (*d*)) was incubated with healthy mouse antero-lateral abdominal wall (*a*) without radiation and (*b*) with 1000 roentgen X-irradiation before incubation. The oxygen uptake of Ehrlich ascites cells was then compared in the media prepared with and without radiation.

(2) As above, except that 0·5 ml. of 0·3 M potassium succinate was added to each 70 ml. of phosphate buffered medium (*d*) either before or after incubation with mouse tissue.

(3) As above, except that Ringer phosphate-succinate medium (*c*), i.e. containing no glucose, was used for incubation with the mouse tissue.

Oxygen uptake of ascites cells was measured in triplicate in each batch of corresponding non-irradiated and X-irradiated media. Experiments were conducted at 37° C. over periods of 40 minutes, during which time respiration remained undiminished. The gas phase was pure oxygen.

Table VIII shows that in phosphate buffer with glucose as substrate, the oxygen uptake of ascites cells was on an average 14·7 per cent lower in the medium incubated with tissue after X-irradiation. In phosphate buffer containing glucose and succinate as substrates, the effect was less pronounced with 4·4 per cent average lowering of oxygen uptake in the X-irradiated medium. Only 1 per cent average lowering was observed in phosphate medium with no glucose but succinate added prior to incubation.

TABLE VIII.—*Oxygen Uptake of Ehrlich Ascites Cells in Phosphate Buffered Media Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Medium and substrate	No. of corresponding batches	No. of experiments	Oxygen uptake Q_{O_2}		Percentage difference
			Not irradiated	X-irradiated	
Phosphate buffered medium (*d*) (glucose)	2	6	4·71±0·31	4·02±0·23*	−(14·7±0·8)
Phosphate buffered medium (*d*) (glucose + succinate)	3	9	5·62±0·31	5·38±0·27	−(4·4±0·3)
Ringer-phosphate medium (*c*) (succinate)	3	9	10·08±0·42	9·99±0·40	−(1·0±0·8)

* Average deviation from mean.

(9) *Oxygen uptake of Ehrlich ascites cells in Ringer phosphate solution incubated for various times with mouse antero-lateral abdominal wall*

The Q_{O_2} of Ehrlich ascites cells was measured in succinate-fortified Ringer phosphate solution (*c*) which had been incubated with healthy mouse antero-lateral abdominal wall for varying times.

The results in Table IX show that, within the limits of experimental accuracy, the Q_{O_2} values of ascites cells were identical in media incubated between 1–5 hours with mouse tissue.

(10) *Oxygen uptake of Ehrlich ascites cell mitochondria in phosphate buffered medium incubated with mouse antero-lateral abdominal wall*

The oxygen uptake of Ehrlich ascites cell mitochondria was compared in 7 corresponding batches of phosphate buffered medium incubated with healthy mouse tissue (*a*) without radiation and (*b*) with 1000 roentgen X-irradiation before incubation. Media were triturated to pH 7·5 with KOH after incubation.

Mitochondria showed a negligible endogenous oxygen uptake in phosphate buffered medium which had not been incubated with mouse antero-lateral abdominal wall and to which no citric acid cycle substrates had been added. However

TABLE IX.—*Oxygen Uptake of Ehrlich Ascites Cells in Ringer Phosphate Solution Incubated for* 1–8 *hours with Mouse Antero-lateral Abdominal Wall. No Irradiation*

Time of incubation (hours)	Oxygen uptake Q_{O_2}	Average Q_{O_2}	Percentage increase above control
0 (Control)	7·1 7·4	7·3	—
1	7·7 8·1	7·9	8.2
2	7·9	7·9	8·2
3	7.6 8·2	7.9	8.2
5	7·9 7·8	7·9	8·2
8	8·2 8·8	8·5	16·5

the mitochondria showed considerable oxygen uptake in the same medium after incubation with mouse tissue. This was apparently due to liberation of oxidisable substrates, from the tissue into the medium during incubation.

The results in Table X show that the oxygen uptake of Ehrlich ascites mitochondria was on an average 20 per cent lower in the medium incubated with tissue after irradiation, than in the control incubation medium prepared without irradiation.

As an additional part of each experiment the biochemical intactness of the mitochondria was checked by measuring their oxygen uptake in phosphate buffered medium fortified with 0·01 M potassium succinate as substrate (cf. Hawtrey and Silk, 1960). Batches showing Q_{O_2} values in the range 85–130 were used for experiment.

TABLE X.—*Oxygen Uptake of Ehrlich Ascites Cell Mitochondria in Phosphate Buffered Medium Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No.	Oxygen uptake Q_{O_2} Not irradiated	Oxygen uptake Q_{O_2} X-irradiated	Percentage difference
1	62·7 —	52·8 56·4	−12·9
2	100·1 93·0	81·0 79·3	−17·0
3	38·7 38·6	30·8 32·8	−17·7
4	41·9 47·5	34·1 32·7	−25·3
5	70·5 96·2	56·8 58·4	−30·9
6	94·9 77·6	80·5 71·7	−11·0
7	76·1 72·0	58·2 47·0	−29·0
Average	70·0±18·5*	55·2±14·4	−(20·5±6·7)

* Average deviation from mean.

(11) *P/O ratio of Ehrlich Ascites cell mitochondria in phosphate buffered medium incubated with mouse antero-lateral abdominal wall*

As part of the experiments to measure O_2 uptake of Ehrlich ascites cell mitochondria, the *P/O* ratios of these particulates were determined in 7 corresponding batches of phosphate buffered medium incubated with healthy mouse tissue (*a*) without radiation and (*b*) with 1000 roentgen X-irradiation before incubation.

The results in Table XI show that the *P/O* ratio was on an average 16 per cent lower in the medium incubated with tissue after X-irradiation. Since no citric acid cycle metabolites were added at any stage in the experiments, the *P/O* ratios shown are those of the substrate(s) liberated from the mouse tissue into the phosphate buffered medium during incubation.

TABLE XI.—*P/O Ratio of Ehrlich Ascites Cell Mitochondria in Phosphate Buffered Medium Incubated with Mouse Antero-lateral Abdominal Wall* (a) *Not Irradiated and* (b) 1000 *Roentgen X-irradiated before Incubation*

Batch No.	*P/O* ratio: Not irradiated	*P/O* ratio: X-irradiated	Percentage difference
1	1·16	1·11	−10·4
	—	0·97	
2	1·13	1·09	−8·1
	1·14	1·01	
3	1·14	1·10	−8·0
	1·11	0·97	
4	1·24	0·84	−26·7
	1·08	0·87	
5	1·29	0·94	−20·5
	1·15	1·01	
6	0·91	0·84	−12·0
	—	0·77	
7	0·98	0·85	−26·4
	1·14	0·71	
Average	1·12±0·07*	0·93±0·10	−(16·0±7·3)

* Average deviation from mean.

(12) *Effect of radiation on the protein and amino acid analysis of media after incubation with mouse antero-lateral abdominal wall*

Table XII shows the total protein content, and the content of free amino acids and peptides present in corresponding batches of various media incubated with mouse tissue. In all cases the incubation medium prepared after X-irradiation showed a lower protein content and a higher free amino acid and peptide content than the corresponding non-irradiated medium.

DISCUSSION

Previous work (Silk, Hawtrey and Macintosh, 1958) showed that irradiation of the normal tumour-bed and stroma cells of a neoplasm, might "indirectly" affect the respiratory metabolism of non-irradiated malignant cells. The "indirect" effect was considered due to radiation induced substances from the normal cells reaching the malignant cells via the common environment of peripheral lymph.

TABLE XII.—*Protein and Amino Acid Analysis of Media after Incubation with Mouse Antero-lateral Abdominal Wall* (a) *without Irradiation and* (b) *with* 1000 *Roentgen X-irradiation Prior to Incubation*

Medium	No. of corresponding batches	Total protein (mg./ml.)			Total free amino acids and peptides (mg./ml.)		
		Not irradiated	X-irradiated	Percentage difference	Not irradiated	X-irradiated	Percentage difference
Ascites plasma incubated with mouse tissue	5	8·85±0·57*	7·83±1·23	−(15·8±12·6)	1·25±0·21	1·30±0·14	+(6·0±15·8)
Ascites plasma deproteinised after incubation with mouse tissue	4	—	—	—	0·88±0·37	0·96±0·14	+(48·7±60·4)
Ascites plasma deproteinised after incubation without tissue	3	—	—	—	0·08±0·16	1·04±0·17	+(26·0±7·7)
Phosphate buffered medium (*d*) incubated with mouse tissue	8	1·02±0·18	0·85±0·21	−(16·6±7·2)	0·45±0·13	0·58±0·17	+(28·6±6·7)

* Average deviation from mean.

For the present investigation, mouse antero-lateral abdominal wall tissue has been selected to represent part of the normal tumour-bed and stroma of the Enrlich ascites tumour. Various physiological media have been incubated with this tissue to provide the environmental fluids through which X-irradiation has been found to influence the respiratory metabolism of non-irradiated ascites cells and mitochondria.

In bicarbonate buffered media, intact ascites cells showed slightly lower aerobic glycolysis (Tables I and III) but markedly higher oxygen uptake (Tables II, IV, V and VI) in the X-irradiated medium than in the corresponding non-irradiated control. No elevation of cell respiration was observed in the case of ascites plasma deproteinised after X-irradiation and incubation in the absence of mouse tissue (Table VII).

In phosphate buffered media, the oxygen uptake of ascites cells was lower in the medium prepared with X-irradiation prior to incubation (Table VIII). The effect is therefore opposite to that found in bicarbonate buffered media. A similar inversion of effect was observed in previous work (Silk, Hawtrey and Macintosh, 1958) and may be due to competition between respiratory and glycolytic processes for the low amount of inorganic phosphate usually available in physiological bicarbonate media (cf. Kvamme, 1958).

The respiration and P/O ratio of ascites cells mitochondria were also found to be depressed in the phosphate buffered medium incubated with mouse tissue after X-irradiation (Tables X and XI). The effect therefore parallels that observed for intact cells in phosphate media, but is again opposite to that observed for whole cells in bicarbonate buffered media.

Due to protein radiolysis, the X-irradiated incubation medium has, in each case, a lower protein content but a higher free amino acid and peptide content than the corresponding non-irradiated medium (Table XII). This holds for X-irradiated and non-irradiated ascites plasma incubated in the absence of mouse tissue, yet the oxygen uptake of ascites cells was not different in the two media (Table VII). Thus radiolysis of the environmental medium itself would not account for the observed effects on respiratory metabolism.

The media incubated with mouse abdominal wall contain respiratory substrates liberated by the tissue. This is evidenced by the high Q_{O_2} of mitochondria in phosphate buffered media incubated without added substrates such as succinate (Table X) and by the fact that ascites cells show a higher Q_{O_2} in Ringer phosphate-succinate solution incubated with mouse tissue, than in the same medium without incubation (Table IX). The amount of substrates liberated from the tissue during incubation times of 1–5 hours was adequate to maintain the respiration rate of ascites cells at a consistent level above the control value (Table IX).

A mere difference in quantity of liberated substrates would not however account for the difference in rates of oxygen uptake shown by ascites cells and mitochondria in corresponding batches of non-irradiated and X-irradiated media. It would therefore seem that the X-irradiated medium contains in addition some radiation induced factor(s) capable of affecting the rate of oxygen consumption. Table VIII shows that liberation of these factors may depend upon the presence of glucose in the medium incubated with mouse tissue.

Bernheim, Barber, Ottolenghi and Wilbur (1958) found that certain tissues with no natural anti-oxidants form peroxides on aerobic incubation, whereas other tissues do this if the natural anti-oxidant is destroyed by radiation. Shuster

(1955) showed that methyl linoleate and linolenate hydroperoxides inhibit the oxygen uptake of Ehrlich ascites cells in Ringer phosphate solution and inhibit their glycolysis in Ringer bicarbonate solution. Such perioxides also inhibit the oxidising enzymes in liver mitochondria (Ottolenghi, Bernheim and Wilbur, 1955).

Our findings concerning the radiation effect on glycolysis in bicarbonate media (Tables I and III) and on oxygen uptake in phosphate media (Tables VIII and X) are therefore compatible with the presence of peroxides in the X-irradiated medium. The elevation of oxygen uptake observed in bicarbonate buffered X-irradiated medium (Tables II, IV, V and VI) does not however appear to be compatible with the known effects of peroxides on respiration. The X-irradiated media were examined for organic hydroperoxides using both iodide and titanous sulphate reagents (Scholes, Weiss and Wheeler, 1956) but none were found present. Spectroscopic examination of corresponding non-irradiated and X-irradiated media revealed no qualitative difference in pattern in the region 200–1800 mμ.

It may thus be concluded that the environmental fluid incubated with an irradiated normal tissue representing the tumour-bed of a neoplasm, contains substances which affect the respiratory metabolism of non-irradiated malignant cells. The factors responsible for this " indirect " effect are at present unknown, but do not result from radiolysis of the environmental fluid itself and must therefore originate from metabolism of the tissue irradiated in contact with this fluid.

SUMMARY

1. Various proteinaceous and non-proteinaceous physiological media were incubated 3 hours at 37° C with mouse antero-lateral abdominal wall tissue (*a*) without radiation and (*b*) with 1000 roentgen X-radiation of the medium plus tissue prior to incubation.

2. The repiratory and glycolytic metabolism of Ehrlich ascites cells and mitochondria was then compared in corresponding batches of media prepared with and without radiation. In some experiments oxidisable substrates were added to the media, while in others substrates liberated from the mouse abdominal wall tissue served to maintain respiration of the added ascites cells or mitochondria.

3. With bicarbonate buffering, intact ascites cells showed lower aerobic glycolysis and markedly elevated oxygen uptake in the X-irradiated medium.

4. With phosphate buffering the opposite effect was observed. Both intact ascites cells and mitochondria showed lower oxygen uptake in the X-irradiated medium. Mitochondria showed lower P/O ratios in the X-irradiated medium than in the corresponding non-irradiated control.

5. Since neither the cells nor the mitochondria were directly radiated, the results demonstrate an " indirect " effect of X-radiation on respiratory metabolism. The effect operates via the environmental medium irradiated in contact with a tissue selected to represent the normal tumour-bed and stroma of the Ehrlich tumour.

6. The " indirect " effect does not seem due to radiolysis of the environmental medium itself, nor do organic hydroperoxides appear to be responsible. It is suggested that metabolic products from the irradiated tissue are liberated into the medium, and that these influence the respiration rate of added Ehrlich ascites cells and mitochondria.

This work has formed part of the research being undertaken in terms of a Senior Fellowship awarded to one of us (M. H. S.) by the National Cancer Association of South Africa.

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THE DEOXYRIBONUCLEIC ACID CONTENT OF CARCINOMA OF THE UTERUS: AN ASSESSMENT OF ITS POSSIBLE SIGNIFICANCE IN RELATION TO HISTOPATHOLOGY AND CLINICAL COURSE, BASED ON DATA FROM 165 CASES

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Received for publication October 31, 1959

RECENT technical advances in chromosome cytology have confirmed the existence of a high degree of constancy in mammalian somatic tissues (Ford, Hamerton and Mole, 1958; Tjio and Puck, 1958) and have served to throw into sharp relief the diversity of chromosome number that has been reported in malignant tumours (Levan, 1956*a*; Ising and Levan, 1957; Manna, 1957; Klein, 1959; Makino, Ishihara and Tonomura, 1959; Richards and Atkin, 1960). The association of certain congenital abnormalities in man with loss or duplication of single chromosomes (Ford, Jones, Polani, de Almeida and Briggs, 1959; Jacobs and Strong, 1959; Lejeune, Gautier and Turpin, 1959) further demonstrates that profound developmental disturbances may result from such changes. This could be inferred from studies on lower animals and plants, but is only now susceptible of direct investigation in man. On the other hand, though some mammalian tumours have an apparently normal chromosome complement (Klein, 1959), the great majority of those for which reliable counts are available present differences in chromosome number from the normal, and from each other, which extend over a wide range. Structural chromosomal changes, also, are common. Relatively little is known however about human tumours as distinct from experimental animal tumours. In the face of the great variation which appears to occur, it is obvious that only by studying a large number of cases can any general pattern be expected to emerge. Only then may it be possible to discover whether or not a correlation exists between the chromosomal changes and the characteristics of the tumour, such as its degree of autonomy, its tendency to invade or metastasize, its degree of differentiation, or tendency to metaplasia, or its sensitivity to ionizing radiations.

Studies on "ascites" tumours of rodents indicate that each tumour strain maintains, within limits, a degree of constancy, and has a modal chromosome number which may not change for many transplant generations; however the fact that changes may occur suggests that they may be a factor in the "progression" of tumours towards greater malignancy, or more specifically towards adaptation to changing environmental conditions. Unfortunately, relatively little is known about the chromosomes of primary (untransplanted) tumours, even in experimental animals. Perhaps the most urgent and intriguing problem is the relation of chromosomal change to the inception of the neoplastic process; nevertheless much may be learnt from the study of fully-developed tumours,

especially in man, where the findings may have a direct relevance to the clinical management of the condition.

Several complementary approaches are often necessary in the attack on any single problem or group of problems : tissue-culture techniques, which have proved so useful in the study of the chromosomes of normal cells (Tjio and Levan, 1956 ; Tjio and Puck, 1958) and of marrow cells in leukaemias (Ford, Jacobs and Lajtha, 1958 ; Baikie, Brown, Jacobs and Milne, 1959), will no doubt be increasingly applied to the study of malignant disease, and much may be learnt. Apart from purely technical difficulties, however, the altered conditions in tissue-culture and the possibility thereby of the selection of certain cell-types from a mixed population may make it difficult to relate the findings to the state of the tumour before its removal from the body. In a previous paper (Atkin and Richards, 1956), we have discussed the application of microspectrophotometry to the study of human tumours : from an estimate of the amount of Feulgen stain, and hence of deoxyribonucleic acid (DNA), in the individual cells of a tumour, data can be obtained that give a measure of its chromosome complement. In a preliminary survey of normal tissues and malignant tumours from various sites, we were able to demonstrate that, while normal cells showed little variation from the diploid value, the interphase cells in the tumour specimens tended to show a greater range of DNA values, partly due to the presence of cells synthesizing DNA and partly to aneuploidy and polyploidy. Nevertheless the majority of cells were grouped around a modal value which varied in different tumours but in most cases was close to either the diploid or tetraploid level. Since the near-diploid tumours frequently had modes which were in fact a little above diploid, it was apparent that by this criterion (i.e. the modal DNA value) the majority of tumours differed from the normal. In a further study based on 14 cases in which we were able to compare directly DNA content and chromosome number for the same tumour (Richards and Atkin, 1960), it was found that the two sets of values were on the whole in agreement, although in several cases the modal DNA content was higher than would be expected if the ratio of DNA content to chromosome number which we had previously found for normal tissues still held. The ratio of DNA to chromosome number exceeded the normal value on an average by 14 per cent, with a range of −6 per cent to +47 per cent.

In this paper are reported the data obtained by microspectrophotometry of Feulgen stain in a series of uterine carcinomata, which will be considered with special reference to their clinical and histopathological features ; in a parallel paper (Richards and Atkin, 1959) the changes in DNA pattern that have been observed in some of these tumours during and following radiotherapy (including some radioresistant cases) are described.

MATERIALS AND METHODS

(a) *Carcinoma of the cervix uteri.*—Of 132 cases, biopsy material was obtained before treatment from 124, while from the remaining 8 cases specimens of local recurrences following radiotherapy only were measured. From 3 of the 124 cases measurements were obtained both from the tumour before treatment and from local recurrences which subsequently developed following radiotherapy. Thus the total number of locally recurrent tumours that were measured is 11.

(b) *Carcinoma of the corpus uteri.*—Measurements were obtained from 33 cases, of which 30 had not previously received treatment.

All the material was obtained under general anaesthesia in the operating theatre; in the untreated cervix cases, biopsies were usually taken immediately before the first Stockholm insertion. Part of the material was retained for cytological studies, including, in suitable cases, chromosome counts; from the remainder, smears were prepared for subsequent measurement of Feulgen stain as previously described (Atkin and Richards, 1956). At the same time, material was sent for routine histological examination. Although there may be relatively few late-stage cases, it is probable that the 124 cervix cases approximate to an unselected series, since biopsies were performed at this hospital on almost every case referred for treatment, including a number of Stage III and IV cases referred for palliative radiotherapy. A few cases (under 5 per cent of the total number) have been excluded from this series because of scarcity or absence of tumour cells in the biopsy material.

In collating our results, derived as before from the measurement of the amount of stain in a random sample of interphase cells, we have been concerned with (*a*) the modal DNA value and (*b*) the variation within each sample. (*a*) *The modal DNA value.*—In order to obtain a numerical value for the DNA mode, the following procedure has been adopted: the average value in arbitrary units of cells that fall within about 15 per cent of the mode is calculated: the data are plotted in the form of a frequency histogram with classes having a spread of about ± 5 per cent, and the 3 adjacent classes with the greatest number of cells are usually averaged. This value is then adjusted to that of the mean of the leucocytes and/or fibroblasts present in the specimen, which are given an arbitrary value of 100 units: this will be referred to as the *basic DNA value.* Since measurements on normal epithelial tissues, including cervical epithelium and endometrium (Atkin and Richards, 1956), have consistently been found to have modal DNA values about 10 per cent greater than the mean value of the leucocytes and other cells of mesothelial origin, it seems that the modal DNA value of epithelial tumours, calculated as explained above, should be related to a normal diploid value of 110, rather than to 100. (b) *The variation in individual samples.*—Histograms of the DNA values of a number of individual uterine tumours have been given elsewhere (Atkin and Richards, 1956; Richards and Atkin, 1959). In this paper we shall confine ourselves to some general observations on the degree of spread of DNA values in the tumour samples.

RESULTS

We will first consider the data derived from the tumour samples obtained before treatment.

(a) *The basic DNA values.*—Excluding 2 cervical carcinomata, which are of special interest and will be considered later, all the tumour samples presented a clear mode. Fig. 1 shows the principal modal, or "basic", DNA value, calculated as described in the previous section, of 122 out of the 124 untreated cervical tumours, and of all the untreated corpus tumours. It will be seen that the cervical tumours fall into 2 distinct groups. Taking the value 156 as the upper limit of the lower group, there are 56 cases in the lower group and 66 in the upper. Furthermore, it can be seen that when the logarithm of the modal DNA value is plotted, as in Fig. 1, each group presents an approximately normal distribution. The lower group, which will be referred to as the "lower ploidy group", has a rather narrower

distribution than the upper, with a coefficient of variation of 9 per cent, and a mean of 121 $\pm$ 11. It will be seen that this mean value is well above the mean leucocyte (*l*) value of 100, and about 10 per cent above the value (110) which, as already explained, we regard as the normal diploid epithelial value. There are in fact only a few cases below 110. In contrast, the mean of the upper group (" upper ploidy group ") is close to the normal tetraploid epithelial value (220), the range is somewhat wider, and there are many hypotetraploid cases as well as some in the hexaploid region (mean = 223 $\pm$ 43 ; coefficient of variation = 20 per cent). Although the numbers are fewer, the corpus cases present differences from the cervix cases which may be significant : there are relatively fewer tetraploid tumours and none in the hypertetraploid-hexaploid region ; on the other hand, there are

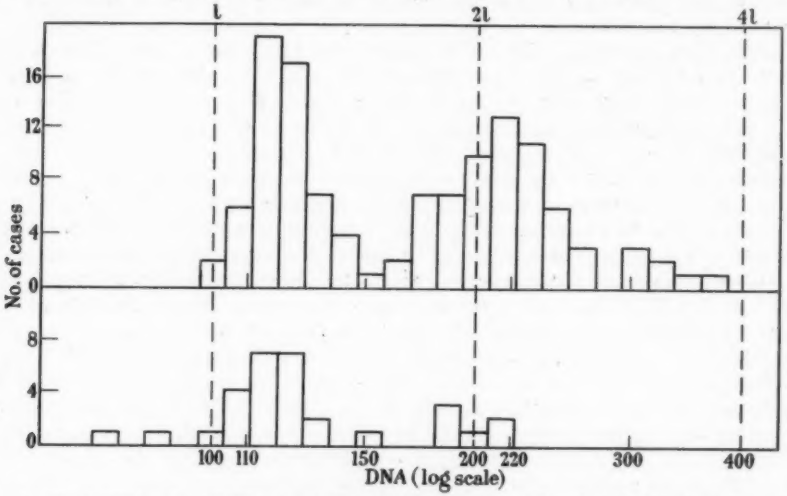

FIG. 1.—Basic DNA value of 122 untreated cervical carcinomata (above), and 30 untreated corpus carcinomata (below).

2 hypodiploid tumours. The mean of the lower ploidy group of the corpus tumours (neglecting the lowest value which is taken as being outside this range) is 118 $\pm$ 13 (coefficient of variation = 11 per cent).

The data are presented in tabular form in Table I. In order to obtain an estimate of the number of tumours which fall close to the normal euploid levels, they have been classified according to whether they fall within $\pm$15 per cent of the normal diploid epithelial value (110) or of twice this value. Seventy-two per cent of the cervical tumours fall within one or other of these limits.

(b) *The degree of spread of DNA values in individual tumours.*—With a few exceptions, this is not very great : in the majority of cases from 50–90 per cent of the cells fall within $\pm$15 per cent of the main mode. There is usually a secondary mode at double the value of the main mode. The relative height of this secondary mode varies in different tumours, reflecting the number of cells that have completed DNA synthesis prior to mitosis, or have achieved a doubling of the basic chromosome complement by abnormal mitosis, endomitosis or endoreduplication.

Taking the 2 principal modes together, we find that in most cases from 70 to 95 per cent of the cells fall within ±15 per cent of either of these limits. To obtain a more precise estimate of the degree of spread, the results from the first 37 consecutive cervical cases were averaged, one case which had a wide spread being excluded: 63 per cent of the cells fell within ±15 per cent of the main mode and 17 per cent within ±15 per cent of the secondary mode. In comparison, 2 specimens of normal cervical epithelium and 2 of normal endometrium in the secretory phase showed 91–96 per cent of the cells within these two limits: primary mode, 76–96 per cent; secondary mode, 0–17 per cent. The degree of spread in the more undifferentiated tumours of the corpus uteri was similar to that in the cervix tumours, but some of the well-differentiated corpus tumours showed less spread, the values falling within the range for the normal tissues just quoted. In a few tumours which had a main mode in the tetraploid region or above, there was a smaller mode at about *half* the value of the main mode; the possible significance of this finding will be discussed later. The cells that fell outside the two main modal ranges only rarely had values much below diploid. In a few tumours, giant cells (i.e. cells having values of from octoploid to 32-ploid or higher) were fairly frequent.

It is necessary to consider to what extent variations in the DNA of the cells, either as regards the position of the basic mode or the degree of spread, may occur in different regions of the same tumour, and therefore to what extent a small sample of tissue is likely to be representative of the whole. Although some degree of variation in any given case cannot be excluded, the rather limited observations that have so far been made on samples from 2 or more regions of the same tumour have revealed no significant differences, except in the relative prominence of the primary and secondary modes.

Having described the variations in DNA content in a series of untreated uterine tumours, we will try to assess the extent to which they can be related to the histopathological and clinical features of the tumours.

TABLE I.—*Untreated Cases Classified According to Basic DNA Value*

Lower ploidy group = up to 156. Upper ploidy group = over 156. Diploid group = 110 ± 15 per cent (94–126). Tetraploid group = 220 ± 15 per cent (187–253).

		Carcinoma of cervix	Carcinoma of corpus
Lower ploidy group	Hypodiploid	0	2
	Diploid	44	19
	Hyperdiploid	12	3
		56	24
Upper ploidy group	Hypotetraploid	10	1
	Tetraploid	45	5
	Hypertetraploid	11	0
		66	6
Wide range (diploid-tetraploid)		2	—
		124	30

Histopathology

In Table II, the histological type of the tumours, classified as before according to the value of the basic DNA mode, is shown. It will be seen that there is no correlation between the DNA level and the degree of differentiation of the squamous cell carcinomata of the cervix, but that all the well-differentiated tumours of the corpus are diploid or hypodiploid. None of the adenocarcinomata or adenoacanthomata of the cervix, however, falls into the diploid or hypodiploid class. Thus there appears to be a significant difference between the squamous cell carcinomata of the cervix, which are frequently near-diploid, and the adenocarcinomata and adenoacanthomata, none of which has a near-diploid mode, although 2 of the adenoacanthomata have a wide range of values, including some in the diploid region. Table II shows that there are 45 squamous cell tumours in the diploid group and 70 in the higher-than-diploid groups, whereas the 17 tumours of the other two histological types are all above diploid or have no clear mode. This difference is highly significant ($p = 0{\cdot}00054$); the limits that we have taken for the "diploid" group are purely arbitrary, but if we narrow the diploid group to ± 10 per cent or broaden it to ± 20 per cent, the differences are still significant ($p = 0{\cdot}0020$ and $0{\cdot}012$ respectively). The adenoacanthomata of the corpus uteri, unlike those of the cervix, are all in the diploid class.

There does not appear to be any correlation between the gross pathological type of the cervical tumours and the basic DNA value. Thus predominantly exophytic tumours occured in both the diploid and tetraploid classes.

Clinical Features

(A) *Carcinoma of the cervix*

(i) *Age of patient.*—The age-distribution of the lower and upper ploidy groups are compared in Fig. 2. There are relatively more younger and older patients, as compared with those in the middle age-groups, in the upper ploidy group, espe-

TABLE III.—*Carcinoma of the Cervix: Age of Patient, at Time of First Treatment, Compared with the Basic DNA Value*

		Under 45 years	45–64 years	Over 64 years
Lower ploidy group	Diploid	9	28	7
	Hyperdiploid	2	6	4
Upper ploidy group	Hypotetraploid	4	5	1
	Tetraploid	15	12	18
	Hypertetraploid	4	4	3
Wide range (diploid-tetraploid)		0	2	0
		34	57	33

cially above the age of 74 where there are 11 cases as compared with 1 in the lower group. From Table III, it can be seen that there is a preponderance of cases in the middle age-groups (45–64 years) in the diploid class, but relatively fewer patients in these age-groups in the tetraploid class. These differences are not

TABLE II.—*Histopathology of the Untreated Tumours and (in brackets) Those in which Specimens of Local Recurrences Only were Measured*

		Carcinoma of the cervix								Carcinoma of corpus					
		Squamous cell Carcinoma			Adenocarcinoma			Adenoacanthoma		Adenocarcinoma			Adenoacanthoma		
Degree of differentiation		Good	Moderate	Poor	Good	Moderate	Poor	Moderate	Poor	Good	Moderate	Poor	Good	Moderate	Poor
Lower ploidy group	Hypodiploid	—	—	—	—	—	—	—	—	2	—	—	—	—	—
	Diploid	2	7	35 (1)	—	—	—	—	—	3	10	1	2	3	(1)
	Hyperdiploid	1	3	7 (1)	(1)	—	—	—	1	—	2	1	—	—	—
Upper ploidy group	Hypotetraploid	1	1	6	—	1	1	—	—	—	1	—	—	—	—
	Tetraploid	3 (1)	8	27 (1)	—	2	2	2	1	—	3 (1)	2 (1)	—	—	—
	Hypertetraploid	—	7	1 (1)	—	—	—	1	2 (1)	—	—	—	—	—	—
Wide range		—	—	—	—	—	—	—	2	—	—	—	—	—	—

statistically significant, however, and would have to be confirmed by observations on further cases.

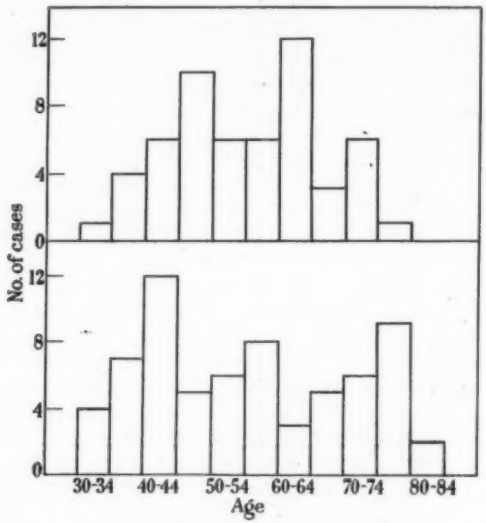

FIG. 2.—Age-distribution of 122 untreated cervical tumours. ABOVE: lower ploidy group. BELOW: upper ploidy group.

(ii) *Clinical stage.*—There appears to be no correlation between the clinical stage and the basic DNA value (Table IV).

TABLE IV.—*Clinical Stage of Cervical Carcinomata*

		Clinical stage				
		I	II	III	IV	Total
Lower ploidy group	Diploid	14	15	10	5	44
	Hyperdiploid	2	5	4	1	12
		16	20	14	6	56
Upper ploidy group	Hypotetraploid	1	2	5	2	10
	Tetraploid	19	12	8	6	45
	Hypertetraploid	3	5	3	—	11
		23	19	16	8	66
Wide range		—	2	—	—	2
						124

(iii) *Response to treatment.*—The great majority of cases were treated by a radical course of radiotherapy: either 3 radium insertions (modified Stockholm technique) or a single radium insertion supplemented by external irradiation with the 4 MeV linear accelerator. A few cases, mostly advanced, received external

irradiation with the linear accelerator or telecobalt unit only. The average duration of follow-up, excluding 32 cases that have died, is 14·6 months, with a range of 0–52 months. Of the 32 cases that have died, 11 were in the diploid class and 12 in the tetraploid. Thus there appears to be no correlation between the DNA value and the survival rate, although most cases have only been followed up for a short time. If however we consider those cases that developed a local recurrence in the cervix or vault of vagina, or were found to have viable tumour tissue in the cervix at subsequent operation, performed at least $2\frac{1}{2}$ months after the first treatment, we find that only one out of 11 had a diploid mode (Fig. 3).

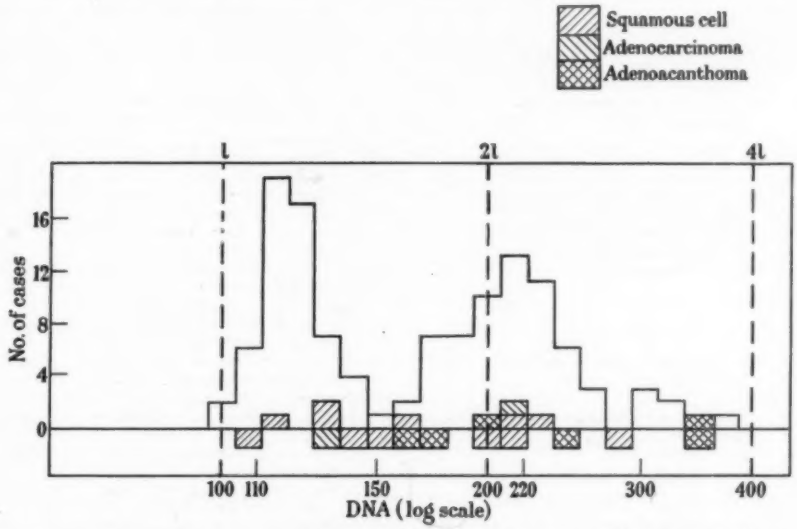

FIG. 3.—Carcinoma of the cervix: radioresistant tumours. The basic DNA value of the untreated tumours which subsequently recurred locally after radiotherapy is shown (shaded) above the baseline; also, below the baseline, are indicated the measurements made on local recurrences. The continuous outline indicates the values obtained for *all* the untreated tumours.

It was possible to obtain measurements on the local recurrences of 3 of these tumours, and of 8 further cases from which pre-treatment biopsies were not available. The DNA values of these local recurrences are indicated below the base-line in Fig. 3. When the data from these cases which showed a poor local response to radiotherapy are compared with the distribution of DNA values of *all* the untreated tumours (indicated by the continuous line in Fig. 3), it can be seen that the former are more evenly distributed through the ploidy ranges, the majority being significantly greater than diploid. The 2 adenoacanthomata that showed a wide spread of values before treatment (not indicated in Fig. 3) gave rise to local recurrences both of which had modes in the triploid region: histograms of these cases, including those derived from specimens obtained during the course of radiotherapy, are illustrated in the parallel paper (Richards and Atkin, 1959). Brief details of the cases that recurred locally are given below.

Case No.	Age-Stage-Degree of differentiation (histology : squamous cell carc. unless otherwise stated)—treatment (St. = Stockholm radium insertion)	Basic DNA value before treatment	Subsequent history (figures indicate time in months after first treatment)	Basic DNA value of local recurrence
32	50 - IV - Poor (adenoacanthoma). 1 St. + DXR	197	No response. 3 : died	—
46	40 - I - Poor (adenoacanthoma). 3 St. + DXR	—	8 : local recurrence. Further DXR (no response)	344
72	59 - II - Poor (adenoacanthoma). 3 St. + DXR	Wide range	Tumour failed to regress. 2½ : laparotomy. Died shortly afterwards	161
78	52 - II - Good. 3 St. + DXR	—	2 : local recurrence. Wertheim's hysterectomy (died post-operatively)	200
105	72 - IV - Poor (adenocarcinoma of cervical stump). Intracav. Ra + DXR	211	10 : died ; primary tumour still present	—
115	58 - III - Poor. Intracav. Ra (ovoids) + DXR	157	3 : local recurrence ; Wertheim's hysterect. 27 : symptom-free	—
319	51 - II - Poor. 3 St.	—	7 : local recurrence ; DXR. Died shortly after	139
427	45 - ? - Good (adenocarcinoma). Total hysterectomy followed by DXR to whole pelvis (4000 r)	—	7 : tumour present in vault of vagina ; further DXR. Subsequently died (tumour still present in vault)	127
480	40 - II - Poor. 3 St.	133	8 : cervix very hard (clinically recurrent growth, but biopsy negative) ; external irradiation (4 MeV linear accel.). 15 : deteriorating	—
502	66 - II - Poor (adenoacanthoma). 3 St.	347	10 : local recurrence ; Ra needle implant—recurrence disappeared. 16 : mass in pelvis	242
537	47 - II - Poor (adenoacanthoma). 3 St.	Wide range	Failed to regress. 2½ : positive biopsy ; DXR. 8 ; died	168
548	39 - I - Poor. 3 St.	—	5 : local recurrence ; Wertheim's hysterect. 13 : recurrent nodule in vagina ; treatment by 4 MeV linear accel.	107
584	43 - I - Poor. 3 St.	217	4 : local recurrence ; Wertheim's hysterect. 10 : vault recurrence and secondaries in scar ; palliative DXR. 12 : deteriorating	—
585	61 - III - Poor. Telecobalt (7000 r)	117	6 : mass side wall of pelvis—but cervix healed. 10 : local recurrence	—
703	45 - I - Poor (areas of mod. diffn.). 3 St.	228	4 : cervix still bulky ; Wertheim's hysterect. (tumour cells present in cervix)	—
756	73 - II - Moderate. 1 St. + radical DXR to pelvis	130	3 : tumour still present in post-fornix	—
824	53 - II - Poor. 4 MeV linear accelerator	—	10 : recurrence in cervix	208
825	32 - III - Poor. 4 MeV linear accelerator	—	5 : recurrence in cervix	148
833	60 - III - Poor. 4 MeV linear accelerator	—	30 : recurrence in cervix	277

(B) *Carcinoma of the corpus*

The age-distribution of the DNA classes for the 30 untreated cases is given in Table V. Sixteen of these cases were treated by an intracavitary Co^{60} source (Strickland, 1953), and their subsequent history is given in Table VI. Owing to

TABLE V.—*Age-distribution of Untreated Cases of Carcinoma of the Corpus Uteri*

		Age						
		45–49	50–54	55–59	60–64	65–69	70–74	75–79
Lower ploidy group	Hypodiploid	1	1	—	—	—	—	—
	Diploid	2	4	4	4	3	1	1
	Hyperdiploid	—	1	—	—	1	1	—
Upper ploidy group	Hypotetraploid	—	—	—	—	1	—	—
	Tetraploid	—	—	1	2	2	—	—

TABLE VI.—*Response to Treatment of 16 Cases of Carcinoma of the Corpus Uteri Treated by Intracavitary Co^{60} Source*

		Subsequent Wertheim's hysterectomy (1–3 months later) —no tumour found on microscopic examination	No evidence of recurrence (no operation performed). Followed up for at least 2 years	Subsequent recurrence in pelvis	Not known
Lower ploidy group	Hypodiploid	2	—	—	—
	Diploid	4	3	1	3
Upper ploidy group	Hypotetraploid	—	—	1	—
	Tetraploid	2	—	—	—

the small number of cases, no deductions can be drawn from these figures, other than that both diploid and tetraploid cases may show a satisfactory response to this mode of treatment.

Details of the 3 cases which had received previous treatment are given below.

Case No. 127.—Total hysterectomy in 1947, when aged 71, for moderately well-differentiated columnar cell adenocarcinoma. Eight years later, polypoid mass in vault of vagina (histological appearance as before), treated by intracavitary radium with good response. Basic DNA value 239.

Case No. 141.—Total hysterectomy followed by DXR to pelvis in 1953; adenoacanthoma of moderate differentiation, aged 56. Two years later: vault recurrence (similar histology); treated by intracavitary radium followed by total vaginectomy. Died 6 months later: secondaries in the abdomen; no tumour found in the pelvis at post mortem. Basic DNA value 122.

Case No. 468.—Treated for carcinoma of the endocervix in 1954 by Stockholm radium technique: moderately well differentiated columnar cell adenocarcinoma; then aged 76. In 1957, treated by total hysterectomy for ? recurrence ? new primary in body of uterus: columnar cell adenocarcinoma, largely anaplastic, with a few areas of moderate differentiation—basic DNA value 225.

DISCUSSION

The distribution of the basic DNA values of the cervical carcinomata (Fig. 1) strongly suggests the presence of two distinct populations; it is reasonable to assume that the tumours in the higher group have undergone a doubling of their

chromosome complement at some stage of their evolution. The central tendency in both groups is clear, and indicates that there is an optimal region, deviations from which are progressively less likely to occur in proportion to their magnitude. The significance of the fact that the majority of tumours in the lower group are hyperdiploid rather than diploid as regards DNA content is not clear. Data on stem-line chromosome counts collected by Ising and Levan (1957) suggest that human tumours in general are quite frequently hyperdiploid, and less often hypodiploid. This is supported by our own somewhat limited observations on the chromosome numbers of the uterine tumours in the present series, counts in the range 48–52 being commonly found (unpublished data). On the other hand, Manna (1957) has found that many human cervical tumours have chromosome modes in the hypodiploid region. It is concluded that more critical chromosome counts are necessary before we can assess the extent to which tumours with near-diploid basic DNA values do in fact differ from the normal in their chromosome complement. If our finding (Richards and Atkin, 1960) of an average of 14 per cent more DNA per chromosome in tumours than in normal tissues were true of cervical tumours in general, we should expect the distribution of modal *chromosome numbers* of the near-diploid tumours to show a peak very close to the diploid number, and not in the hyperdiploid region. However, our data, based on a small series of cases in which it was possible to compare DNA content with chromosome number for the same tumour, probably do not justify such a conclusion ; indeed they suggest that the discrepancy between DNA content and chromosome number is on the whole greater for the tumours with higher chromosome numbers, and minimal for the near-diploid group.

The tumours in the upper group show a distribution that is centred on a value less than twice that of the lower group, and moreover have a rather wider range extending from the triploid to the hexaploid or hypo-octoploid region. Does this represent a similarly wide range of chromosome numbers ? Bader (1959), in the course of microspectrophotometric measurements of DNA in a small series of human ovarian carcinomata, found in one case a discrepancy between the cells in anaphase, which fell into the diploid range, and those in interphase, which had a mode in the tetraploid region, and has suggested that tumours which are " tetraploid " as regards DNA content may not necessarily have a near-tetraploid chromosome number, since " a combination of diploid cells having doubled amounts of DNA and tetraploid cells having the tetraploid amount of DNA would result in a distortion of an interphase frequency distribution relative to the anaphase frequency distribution ". We have already pointed out that some of our tumours, which have their main mode in the tetraploid or hypertetraploid region, have in addition a smaller mode in the diploid-hyperdiploid region. Cytological studies on aceto-orcein squash preparations (Atkin and Ross, unpublished) indicate that these particular tumours are usually characterized by a high incidence of endomitosis, as also may be those that have a basically hyperdiploid mode plus a prominent hypertetraploid secondary mode ; furthermore, although the chromosomes in these tumours are frequently crowded, sufficiently accurate counts have been possible to indicate that cells with near-diploid and near-tetraploid chromosome complements can reach an apparently normal metaphase stage. It is not known, however, whether the tetraploid cells can complete mitosis. It may be, therefore, that the DNA mode is not representative of the chromosome number of the stem-line in all cases ; indeed, if there are *two* classes of *dividing* cells, one

having twice the chromosome complement of the other, it seems quite likely that their relative frequencies cannot be deduced from the relative heights of the corresponding DNA modes. However, the tumours which have a lower secondary mode are in the minority (about 25–30 per cent of those tumours whose basic DNA value lies between 220 and 260), and for most tumours which are " tetraploid " as regards DNA content, the photometric findings are consistent with the cytological observations : chromosome counts, average nuclear size, and, where sex chromatin is evident, the presence of 2 sex chromatin bodies per nucleus (Atkin, 1960).

The occurrence of endomitosis in the hyperdiploid-hypertetraploid group of tumours, which includes those hypertetraploid tumours which do not have a hyperdiploid secondary mode, suggests that these tumours may be in the process of, or may have completed, a transition from the lower to the higher ploidy. Whether cells with a doubled chromosome complement can take over as a new stem-line will of course depend on whether they are capable of normal mitosis. The greatest significance of polyploidy, however, may be that it forms the basis for further evolution. Levan (1956*b*) has pointed out that " tetraploidy in mouse tumours is often only a transient stage on the way to hypotetraploidy . . . secondary numerical variation is often superimposed on the chromosomal doubling ". Although this may well be true for human tumours also, there is no direct evidence for this at the moment. The uterine tumours with hypotetraploid DNA modes do not show much endomitosis, nor do they have lower secondary modes ; their mode of evolution is at present unknown.

In view of the above considerations, it is not surprising that no clear-cut correlation appears between the clinical and histopathological features of the tumours and their DNA values. It might be that those tumours which deviate markedly from the diploid or tetraploid levels (i.e. those in the triploid or hexaploid region) could be regarded as genetically " unbalanced " and be expected more often to show anomalous behaviour. However, we encounter the difficulty that their numbers are fewer, and that they form a continuous series merging into the more nearly " euploid " tumours, so that any classification for statistical purposes must be purely arbitrary.

The perhaps significantly higher incidence in the cervical tumours of high basic DNA values at the extremes of the age-range may reflect a greater tendency towards polyploidization, perhaps due to hormonal stimuli, in the younger and older patients as compared with those in the middle age-groups. It is clear that there is no correlation in the squamous cell cervical tumours between the degree of differentiation and ploidy level. This lack of correlation has also appeared in data (unpublished) on squamous cell tumours from other sites, including vulva, larynx and tongue, where well-differentiated tumours are relatively commoner. On the other hand the figures for the corpus tumours suggest that here there may be a correlation between degree of differentiation and ploidy, since all the well-differentiated tumours are either diploid or hypodiploid ; it may be that the degree of correlation between ploidy and differentiation varies in tumours of different histological type. Perhaps therefore the finding that the adenocarcinomata of the cervix have predominantly high basic DNA values, in the small series of cases that we have observed, may be related to their more or less undifferentiated character rather then to any other factor.

The adenoacanthomata of the cervix require further consideration. None of

these had a diploid DNA mode, although before treatment two of them had a heterogeneous population (as regards DNA values) which included a number of cells in the diploid region. After irradiation therapy, however, in both these cases there emerged an actively-growing strain of cells having a triploid DNA mode. A further case, from which a pre-treatment specimen was not obtained, gave rise to a local recurrence which proved to have a main mode in the hexaploid region, with a smaller triploid mode. The rather frequent occurrence of triploid/hexaploid DNA modes in those adenoacanthomata which proved markedly radioresistent appears to be of particular interest. The possibility of a link between the histogenesis of these tumours, which has been discussed by Glücksmann and Cherry (1956) who also find that they frequently respond poorly to radiotherapy, their apparently anomalous DNA content and often radioresistant character would appear to merit further investigation.*

The data from the squamous cell carcinomata of the cervix that subsequently recurred locally and the measurements actually made on local recurrences (Fig. 3) indicate that these tumours are often higher than diploid, although the difference in distribution from that of the tumours which responded satisfactorily is not statistically significant. It is clear that there is no simple correlation between radiosensitivity and basic DNA level. Perhaps however with an increase in chromosome number there is the increased possibility of variation, which in some tumours may lie in the direction of greater radioresistance.

SUMMARY

1. The DNA content of tumour cells from 132 cases of carcinoma of the cervix and 33 cases of carcinoma of the corpus has been estimated by microspectrophotometry.

2. In all except 2 of the cervical tumours, a clear mode was apparent in the frequency distribution of DNA values of a random sample of interphase cells: in most tumours, from 50 to 90 per cent of the cells fell within ± 15 per cent of a modal value, which has been referred to as the *basic DNA value*.

3. The basic DNA values of individual tumours were found to extend over a wide range, but fell into 2 main groups centred on the hyperdiploid and tetraploid levels respectively. This distinction into 2 groups was clearly seen in the cervical tumours although individual tumours ranged from diploid almost to the octoploid level. There were relatively fewer corpus tumours in the tetraploid region and none with DNA values above tetraploid; on the other hand there were 2 hypodiploid tumours.

4. The relation of the basic DNA value to the chromosome number of the tumour cells is discussed, and it is concluded that in the majority of cases the DNA value bears a reasonably close quantitative relationship to the modal chromosome number, although from evidence obtained in a previous study a strict parallelism is not necessarily to be expected. There is evidence that some of the cervical tumours may be in the process of transition from a hyperdiploid to a hypertetraploid DNA value by means of endomitosis or some other chromosomal-doubling process.

* Measurements have recently been obtained on 3 further adenoacanthomata of the cervix. Two had basic DNA values of 131 and 153 respectively before treatment. A specimen from the third case was not obtained before treatment, but one taken 7 days after the first Stockholm insertion had DNA modes in the triploid and hexaploid regions.

5. There is no significant relation in the cervical tumours between basic DNA value and clinical stage or age of the patient, although there is a suggestion that tetraploid tumours may be relatively commoner at the extremes of the age-range.

6. There does not appear to be a correlation between the degree of differentiation of the squamous cell cervical tumours and their basic DNA value. All of the 7 well-differentiated corpus tumours however are either diploid or hypodiploid, but the number of cases is not large enough to demonstrate a statistically significant correlation. There is a significant difference between the squamous cell tumours of the cervix, which are frequently near-diploid, and the adenocarcinomata and adenoacanthomata of the cervix, none of which has a near-diploid mode.

7. Data derived from (i) untreated cervical tumours which subsequently recurred locally and (ii) measurements made on local recurrences suggest that radioresistant cell strains are more often higher-than-diploid than near-diploid. In particular, several adenoacanthomata which responded poorly to radiotherapy had tetraploid, triploid or hexaploid modes, the two latter being relatively uncommon among the squamous cell tumours.

This work has been made possible by the collaboration of the clinical staff of Mount Vernon Hospital which is gratefully acknowledged, as is the technical assistance of Miss M. Penny. Thanks are due to Dr. Honor B. Fell, F.R.S., Dr. M. H. F. Wilkins, F.R.S., and Dr. Alma Howard, who kindly read the manuscript, and to Dr. J. Boag for statistical advice. Expenses incurred in this work have been defrayed by a grant from the British Empire Cancer Campaign.

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DNA CONTENT OF HUMAN TUMOURS: CHANGE IN UTERINE TUMOURS DURING RADIOTHERAPY AND THEIR RESPONSE TO TREATMENT

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Received for publication October 31, 1959

IONIZING radiations are widely used in the treatment of cancer in man and frequently they produce excellent results. Sometimes, however, cases are encountered which give very poor results although they are of a type that can be expected to respond well to radiotherapy. Although these cases are in the minority (a few per cent of the total) they remind us that response to radiotherapy can vary considerably between different tumours in any one histopathological group. An additional complication is that the immediate response, viewed clinically or histologically, does not necessarily reflect the ultimate success or failure of the treatment. These problems, both practical and biological, have been clearly annotated by Merrill (1958) in a review of the various attempts that have already been made to classify radiation response in terms of "radiosensitivity" and "radiocurability". A great need still exists, therefore, for an *objective* method for predicting radiosensitivity and radiocurability; the search for such a method is impeded in no small degree by the lack of understanding of the effects of radiation on the fundamental processes of dividing cells.

For several years we have been measuring the DNA contents of human tumours both before treatment (which has formed the basis of a previous report (Atkin and Richards, 1956)) and in some cases during radiotherapy and, if possible, at later stages such as at operation. Most of our data refer to uterine tumours partly because they are common and partly because the type of radiotherapy which they receive frequently makes it possible to obtain biopsies at several intervals during the course of treatment. We have now collected sufficient results to warrant some account of the types of effect of the radiation treatment on the pattern of DNA values of the tumours and to consider these results with a view to obtaining a quantitative cytochemical method for assessing radiocurability.

In addition to their practical interest, these observations are of value in the study of the effects of irradiation on a dividing cell population. An extensive literature on this subject already exists but it contains few instances where the effects on the fundamental chemical processes, such as DNA synthesis, have been examined at the single cell level. Our contribution is at a considerable disadvantage compared with an experimental study because we have no "controls", and the factors of the irradiation treatment are those dictated by the clinician. In particular we must accept the fact that the dose received at any point in the tumour is difficult to assess and standardize. Nevertheless, as we have previously suggested

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(Atkin and Richards, 1956), the information that can be obtained from human tumours may be of more direct relevance to the study of this disease in man than perhaps much that is obtainable from studying transplantable tumours of animals. The relative advantages of investigating spontaneous and transplantable tumours have recently been considered by Scott (1958).

MATERIALS AND METHODS

The details of our methods of preparation of tissues and measurements of Feulgen stain have been described previously (Atkin and Richards, 1956). One improvement that has been made since that report, is that the tumour material is now fixed by methanol freeze substitution at the hospital, thus avoiding the delay incurred in transport to King's College before fixation. Feulgen staining is also done at the hospital and the Feulgen-stained specimens are transported mounted in non-drying immersion oil, after having been dehydrated in the alcohol series and passed through xylene. If necessary the stained preparations are stored for short periods at 2° C. Immediately before measurement specimens are rehydrated and mounted in glycerol. This is necessary because the cell-crushing procedure, which is part of the measuring technique, does not work satisfactorily with immersion oil as a mountant. The amounts of Feulgen stain per cell nucleus were measured with the scanning photometer (Deeley, 1955).

The results for the DNA content (amount of Feulgen stain) for 60–100 tumour cells in each tumour are plotted as frequency histograms. In some cases the scale of the amount of DNA is logarithmic instead of linear because some specimens, particularly after irradiation, show ranges of DNA values over several multiples of ploidy. The method that we previously used for calibrating the amounts of stain, so that different specimens may be compared, was also used here: in this a sample of 10–30 inflammatory cells, usually polymorphonuclear leucocytes, is measured in every specimen; these act as a standard which we designate the 'l' value and the scale of amounts of DNA in the histograms is thus in terms of l.

RESULTS

The limitations of any system of classification of human tumours are emphasized by the differences in response of the tumours in any one group to the same method of radiation treatment. This variation in response between individual cases may appear in the early stages of treatment or only after completion of treatment. Likewise, in the results reported here, the effect of irradiation on the DNA content and pattern of DNA values in tumours may vary between individual tumours in both early and late stages of treatment.

The results described here are limited to those on tumours of the uterus for the reasons given in the Introduction. In a parallel paper (Atkin, Richards and Ross (1959)) we have considered our results, both those presented here and from other tumours, in relation to the system of clinical staging that has been standardized for some years, and also to other factors, e.g., age of patient. In this report we have attempted to discuss the changes produced by the radiation treatment in the pattern of DNA values in all the cases in which we have been fortunate enough to have obtained specimens before, during and occasionally after the course of treatment. Table I includes the relevant data on pathology and treatment for the cases which we have selected for illustration in this paper.

TABLE I.—*Table of Data for Cases Described*

Case No.	Age	Site	Stage	Pathology	Treatment	Subsequent history	Fig. No.
501	48	Cervix	I	Poorly differentiated squamous cell carcinoma (P.d.sq.c.ca.)	2 Stk.* + Wert.*	Died from widespread metastases 4 months later	1
491	51	Corpus	—	Moderately well differentiated columnar cell adenocarcinoma	1 Stk. + total hysterectomy	Well 8 months later	2
161	73	,,	I	Moderately well differentiated columnar cell papillary adenocarcinoma	Intracav.$Co^{60} \times 2$	Well 41 months later	3
421	43	Cervix	I	Moderately well differentiated keratinizing squamous cell carcinoma (exophytic)	3 Stk.	Well 19 months later	4
449	58	,, (stump)	II	P.d.sq.c.ca.	Ra. ovoids (60 mg. × 30 hr.) Wert.	Well 9 months later	5
486	33	Cervix	I	P.d.sq.c.ca. with slight keratinization	3 Stk.	Well 21 months later	6
280	51	,,	II	Keratinizing sq.c.ca.	Ra. (modified Paris technique) + Wert.	Recurrence left side of pelvis 12 months later	7
72	59	,,	II	P.d. adenoacanthoma	3 Stk. + DXR to parametria	2 months later, colostomy (mass in pelvis and local recurrence). Died a few weeks later	8
537	47	,,	II	P.d. adenoacanthoma	3 Stk.	2 months later at laparotomy : inoperable tumour invading rectum : course of DXR to pelvis. Subsequently developed local recurrence in cervix and died 8 months after first treatment	9

* Stk. = Stockholm radium insertion.
* Wert. = Wertheim's hysterectomy.

The cases have been classified into groups according to the type of change shown in the pattern of DNA values. Out of a total of 29 cases where specimens were obtained during treatment, 6 showed only very slight evidence of change shortly after the first radiation treatment (3–7 days), 20 cases showed an effect that for various reasons may be regarded as a typical response to irradiation, and the remaining 3 cases, although they gave the typical response at first, had several features which suggested that they were unusual. It is interesting that 2 of the 3 unusual cases were similar in certain important respects. In the following account we shall describe examples of each of the above categories.

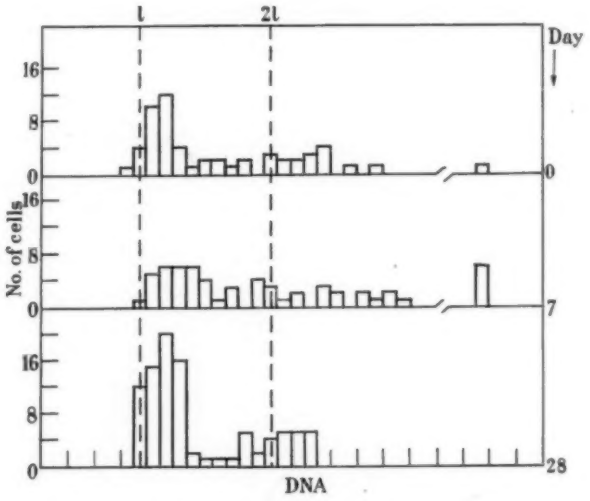

FIG. 1.—Carcinoma of the cervix: before treatment; 7 days after first radium insertion; at operation 4 weeks after first treatment.

1. *Slight change*

Although the conditions of irradiation of the tumours differed somewhat in detail according to the individual needs of the case the differences do not seem to be sufficient to explain the fact that 6 out of 29 cases show little, if any, early change in their pattern of DNA values, in contrast to the marked changes seen in the remaining cases.

An example of this type of case is given in Fig. 1 (case No. 501) which is a Stage I cervical carcinoma. The basic DNA value (i.e., the post-telophase DNA value of most of the tumour cells which is usually indicated by a prominent mode near the lower limit of the histogram) is seen to be approximately the same for all three tumour samples. Although there are slight indications of change at day 7, such as a reduction in the height of the primary mode and increase in the frequency of higher DNA values, the pattern at day 28 is that of a typical dividing cell population. This impression is strengthened by the fact that all stages of mitosis were observed in parallel aceto-orcein preparations. It is noteworthy, however,

that this case received only 2 Stockholm doses, the treatment being completed by operation.

Fig. 2 and 3 show two cases of carcinoma of the uterine body (case No. 491 and 161) which were treated by radium and by cobalt (Co^{60}) insertions (Strickland,

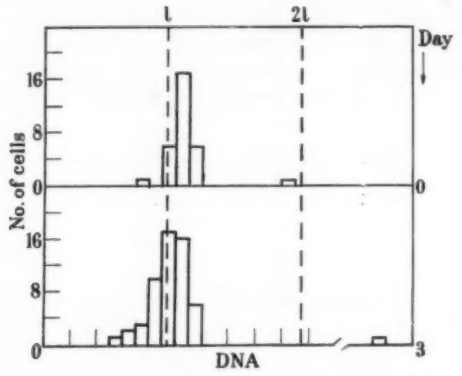

FIG. 2.—Carcinoma of the corpus: before treatment; 3 days after radium insertion.

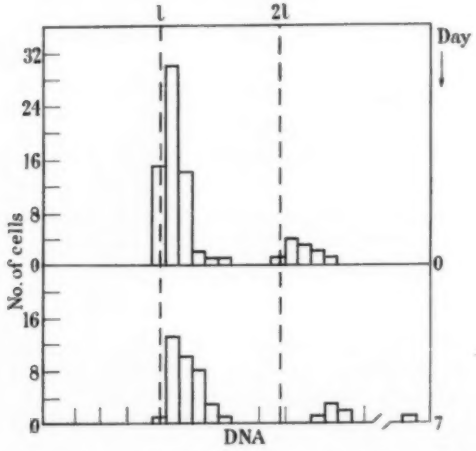

FIG. 3.—Carcinoma of the corpus: before treatment; 7 days after first treatment with intracavitary Co^{60}.

1953) respectively. In neither case can any significant change be detected at the short times after irradiation at which second specimens were available but in both these cases the specimen may have been taken too soon after the beginning of irradiation for marked changes to have occurred.

Although all 6 cases, of which the aforementioned 3 are examples, failed to show significant changes in tumour samples taken during treatment, the clinical

response in each case was good, except in case No. 501 where the presence of mitoses in the Wertheim specimen indicated that active tumour remained, but since 4 of these cases (including No. 501) were operated upon (total hysterectomy or Wertheim's hysterectomy) at varying times following radiotherapy, the success of the radiation treatment alone could not be assessed.

2. *Typical changes*

Apart from the 6 cases mentioned in the previous section, all cases studied showed significant changes in the early stages of treatment (i.e. at 7 days). These

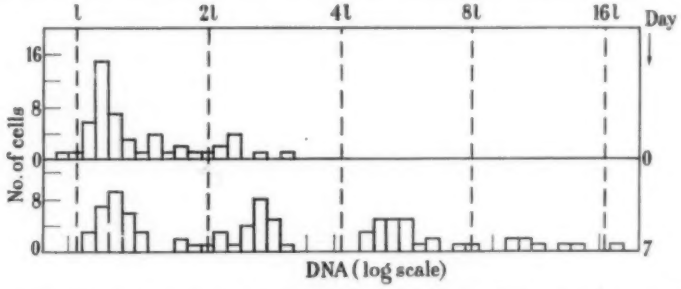

FIG. 4.—Carcinoma of the cervix : before treatment ; 7 days after first radium insertion.

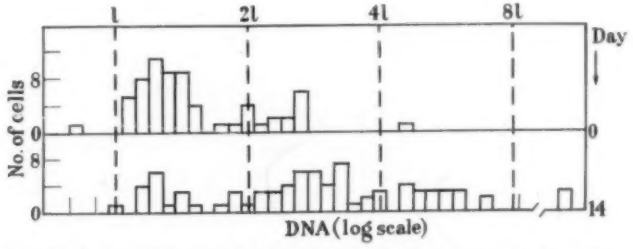

FIG. 5.—Carcinoma of the cervix (stump) : before treatment ; 14 days after first radium insertion.

changes are regarded as typical of the early effects of irradiation on the pattern of DNA value because they appear in most tumours irrespective of additional or subsequent changes (or of the final outcome of the treatment). These changes are to some extent similar to those that have been observed as radiation effects on DNA content in the cells of transplantable animal tumours (see later).

Case No. 421 (Fig. 4), an exophytic Stage I cervical carcinoma, shows a striking accumulation of cells with larger DNA values, and, in this case, the histogram has definite modes at exact multiples of the basic DNA value. This is a characteristic pattern which is frequently seen at 7 days following the first radiation treatment. These higher values are also commonly found at 14 days after a radium insertion, as for example, in Fig. 5 (case No. 449, a Stage II cervical tumour). The appearance of higher multiples of DNA values is to be expected from the well-known "giant cell" formation after irradiation seen in histological studies.

Both cases described so far in this section were near-diploid tumours, but the typical radiation response of increasing frequency of higher ploidy multiples is also found with near-tetraploid (2 *l*) tumours. An example of this is case No. 486 (Fig. 6), a cervical carcinoma, which at day 7 after the first radium insertion shows a prominent mode at near-octoploid (4 *l*) and also values in excess of 16 *l*. Seven days later (after a second insertion of radium at day 7) higher multiple values persist but there is some suggestion of a mode at the original basic DNA value.

In all 3 cases so far described the last histogram represents the last occasion on which viable cells were present in samples taken during treatment; subsequent samples, where available, contained no tumour, and 6 months follow-up examina-

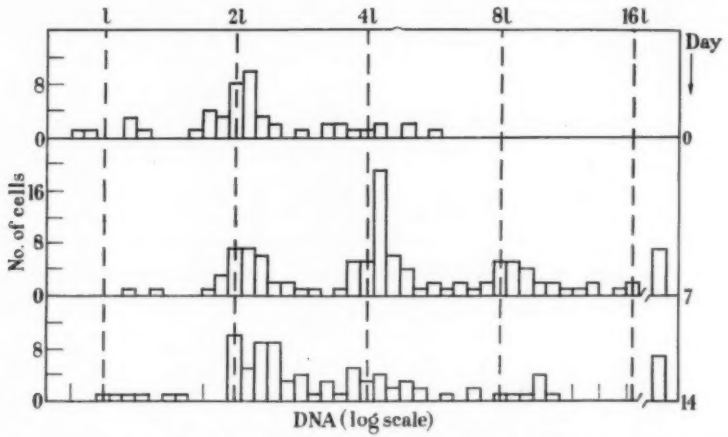

FIG. 6.—Carcinoma of the cervix: before treatment; 7 days after first radium insertion; 7 days after second radium insertion.

tions gave favourable results. The typical changes seen in the early stages of radiation treatment probably reflect only the sensitivity of the majority of the tumour cells to radiation and do not imply that the tumour as a whole is curable, because such response changes are found in both successfully and unsuccessfully treated cases.

3. *Unusual changes*

Some of the cases that we have had the opportunity to examine have been noteworthy for one of two reasons. Firstly, they may have shown unusual features in the changes in their pattern of DNA values brought about by the radiation treatment, or secondly, they may have shown radioresistant properties which make them important from the clinical standpoint. Clearly, our aim is to see whether we find cases that are noteworthy for both reasons, or better still, if those that have a poor prognosis have patterns of DNA values *before treatment* which are unusual. In this way it might be possible to obtain an objective quantitative criterion of radioresistance. We shall now describe some of the cases in which

we have encountered unusual results and later attempt to assess the value of our observations with respect to such a criterion.

An interesting case was one treated at the Middlesex Hospital by a modified Paris radium technique in which it was possible to obtain a biopsy very much sooner after the beginning of treatment than the customary 7 days (for the Stockholm method). This was a Stage II cervical tumour, the results for which

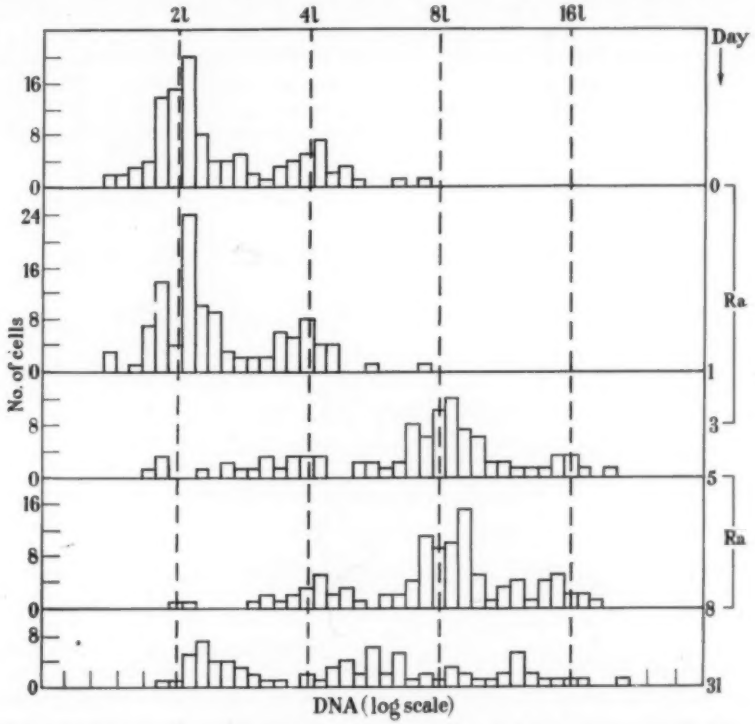

FIG. 7.—Carcinoma of the cervix : before treatment ; 24 hours after commencement of treatment ; 5 days after commencement of treatment ; at completion of radiation treatment ; at operation 31 days after commencement of treatment (radium was inserted for two periods of 3 days).

are illustrated in Fig. 7 (case No. 280). Radium insertions were given for two periods : day 0 to day 3, and day 5 to day 8. It is noteworthy that the change in the pattern of DNA values towards the appearance of higher multiple values is not found at 1 day after the beginning of irradiation, whereas when 5 days have elapsed, during three of which the tumour was exposed to radiation, there is a very marked change in this direction. The patient underwent a Wertheim's hysterectomy at day 31 when the small residuum of tissue found indicated that the tumour was radiosensitive. After 4 months, however, a recurrence appeared in the left side of the pelvis.

As already mentioned we have found two cases in which the radiation treatment seems to have had a more interesting effect on the tumour than the typical one of increasing the frequency of cells with higher DNA content; the typical effects in the early stages are nevertheless present. The unusual feature is the fact that the pattern of DNA content in the tumour after the end of radiotherapy differs from that present in the untreated tumour in a manner not found in other cases. The first of these is given in Fig. 8 (case No. 72—a Stage II cervical carcinoma).

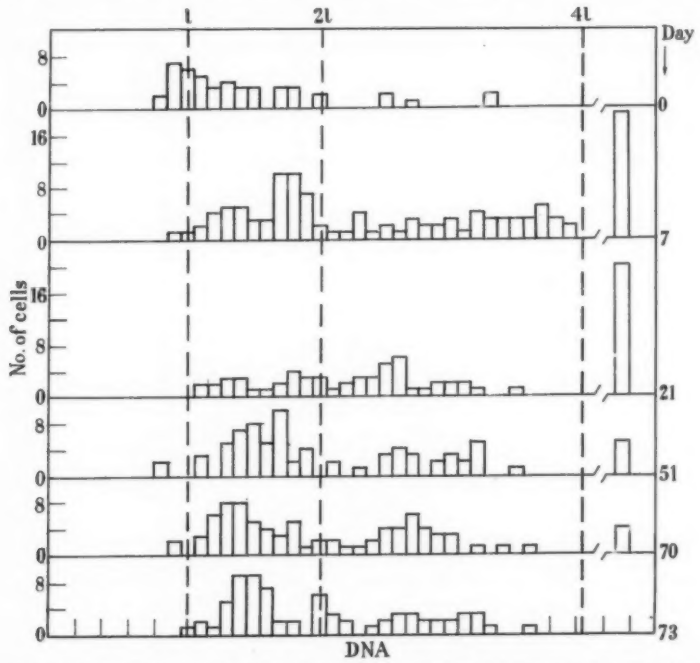

FIG. 8.—Carcinoma of the cervix (adenoacanthoma): before treatment; 7 days after first radium insertion; 14 days after second radium insertion; biopsy specimens of actively-growing tumour at primary site obtained on days 51, 70 and 73.

In this case, the pre-treatment biopsy is itself unusual with a relatively flat distribution; the basic DNA value is difficult to determine owing to the lack of a definite mode but is probably very near the l value. The tumour failed to regress after radium treatment by a modified Stockholm technique so that tumour material was available at day 7 and 21 during radium treatment, at day 51 and 70 after treatment, and finally at day 73 when laparotomy was performed. Soon after the last operation the patient died.

The samples at day 7 and day 21 both show the typical increase in the frequency of higher DNA values of which, however, relatively few exceed 4 l; cell divisions were noted in each sample, and anaphases and telophases were seen at day 7 (i.e., very soon after irradiation). Almost 2 months after the beginning of

treatment there was actively growing tumour at the primary site; this shows a fairly definite mode of DNA values at about $1\frac{1}{2}$ *l*. In both subsequent tumour samples this mode is very pronounced, and there is a range of values going up to but rarely exceeding 3 *l*. This pattern of DNA values differs very much from that of the original tumour and hence it appears that a new actively growing tumour strain had emerged. The radiation treatment may have selected a resistant cell lineage having a different DNA content from the majority of the original tumour cells.

Many of the unusual features of this case (No. 72) were also found in another (case No. 537—Stage II cervical carcinoma), the DNA values for which are given in Fig. 9. Unfortunately, biopsies were available at day 0, 7 and 81 only. In the pre-treatment sample (day 0) an extremely wide range of DNA values is found,

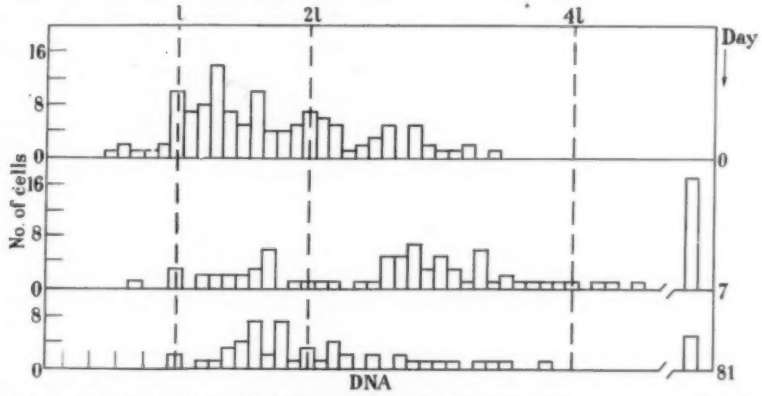

FIG. 9.—Carcinoma of the cervix (adenoacanthoma): before treatment; 7 days after first radium insertion; biopsy specimen of local recurrence on day 81.

i.e. well outside the twofold range in which most of the cells of a dividing cell population usually lie. Like the previous case the pre-treatment distribution is essentially flat, but here the total range of values is much wider than twofold. Although values near *l* are frequent, it is not possible accurately to assign a figure for the basic DNA value. Seven days after the first radium insertion the characteristic increase in the frequency of higher values has occurred but at day 81, when laparotomy was performed, there is evidence of a mode of values appearing between *l* and 2 *l*. There are some indications, therefore, that the changes in the pattern of DNA values following treatment are similar to those found in the case last described, while a comparison of the clinical features and prognosis of the two cases reveals many similarities. Shortly after the end of radiation treatment a considerable amount of tumour remained. In this case (No. 537) the tumour remained inoperable and, moreover, proved resistant to a further course of deep X-rays. It is interesting that both these tumours which had been dramatically unresponsive to radiation treatment and which showed a pattern of DNA values unlike that seen for other tumours, should have been identified as "mixed" tumours according to the histological classification of Glücksmann and Cherry (1956). These authors also find such tumours to be frequently radioresistant.

DISCUSSION

Radiotherapeutic techniques have achieved a considerable measure of success in the treatment of many types of human tumours. It has long been known that ionizing radiations have a greater immediate effect on reproducing cells than on differentiated cells. In this respect, however, radiation probably affects tumour and normal dividing cells in the same manner. The success of the techniques, therefore, has depended largely on the ability of the radiotherapist to design the exposure to radiation in a way that the dose received by adjacent normal tissue is minimal and the maximum dose is delivered to the malignant cells.

Despite the substantial research effort which in recent years has been directed to the study of radiation effects both immediate and long-term (see, for example, Gray, 1956; Scott, 1958; Errera, 1959), comparatively little is known about the effects of ionizing radiation on cells in terms of disruption of the vital chemical processes. One of these vital processes is the synthesis of DNA. This has been shown by many authors (Swift, 1950; Howard and Pelc, 1951; Walker and Yates, 1952; Richards, Walker and Deeley, 1956) to occur during the interphase of the mitotic cycle, and X-ray doses of about 1250–2000 r affect the rate of this synthesis. As judged from isotope studies on animal tumours, regenerating liver and normal tissues, there is a reduction of the rate which, after a single exposure, may last for up to about 40 hours (Howard, 1956; Holmes, 1947; Kelly *et al.*, 1957; Nygaard and Potter, 1959). This effect is temporary, therefore, and the cells can recover, complete their DNA synthesis and pass through a normal mitosis. Complete cessation of DNA synthesis usually requires very much higher doses than 2000 r.

Very small doses of radiation can cause mitotic delay. The results of Caspersson, Klein and Ringertz (1958) have shown very clearly that doses of 1250 r on certain animal ascites tumours while not preventing DNA synthesis, although there may be a temporary cut in the rate, cause an accumulation of cells at the pre-mitotic DNA content. After recovery from irradiation these cells probably pass through a normal mitosis. It is noteworthy, however, that one of the typical effects which we noted in our results, namely, the "doubling-up" of pre-mitotic cells by abortive mitosis with nuclear reconstruction without cell cleavage, endomitosis or endoreduplication, did not appear to occur under the experimental conditions used by Caspersson *et al.* (1958). Twenty-three out of 29 cases of human tumours showed this "doubling-up" which probably had occurred more than once in those cells which later appeared with DNA contents 4 or 8 times higher than the basic DNA value of the tumour. This difference may be explained simply by the possibility that human tumour cells can undergo endomitosis more readily than the animal tumour cells, but the conditions of the irradiation (dose and dose rate, state of oxygenation, etc.) were certainly not the same for the animal tumours as for our clinical cases, so that the difference might be due to one or more of several possible factors. It is noteworthy, however, that the majority of the cases described here showed similar changes in the early stages of treatment despite the fact that the conditions of irradiation could not have been the same for all cases.

The most interesting of the cases which we described here were those (2 out of 29) which showed a high degree of radioresistance (i.e., lack of regression and absence of widespread necrosis) and were in fact incurable by radiation treatment;

their clinical and pathological features were remarkably similar. The microspectrophotometric observations that we made suggest a possible reason for this radioresistance; comparison of the pattern of DNA values in the pre-treatment biopsies with that in biopsies taken during or after treatment provide strong evidence that a selection of cells has occurred. Before treatment the histogram is flat, being without any definite mode and in one case (No. 537) the range of DNA values is sufficiently wide to accommodate much aneuploidy; in addition, both cases show histological evidence that the tumours were composed of more than one type of cell. The slides of these tumours were kindly studied by Dr. Glücksmann who found them to be "mixed tumours" according to his criteria (Glücksmann and Cherry, 1956). After treatment a definite mode of DNA values appeared at an intermediate position between l and $2\,l$, suggesting that the tumours now consisted mainly of one type of cell (with respect to DNA content) being the progeny of a strain of cells which might have been selected out of the original mixed population by virtue of a greater radioresistance than their neighbours. In the absence of competition and perhaps because they were able to use the products of necrosis of killed cells, a phenomenon that has been observed in experiments on animal tumours (Révész, 1955, 1958), this strain of cells gave rise to recurrent tumour.

The most urgent question arising from our results is whether or not they allow such radioresistant tumours to be distinguished from others before their method of treatment is decided. Certainly the pre-treatment pattern of DNA values is unusual in lacking a definite mode but this is not sufficient evidence, nor are our own results based on a sufficiently large number of cases at present, to recommend that a patient be treated other than by radiotherapy, if for clinical reasons this is considered to be the method of choice.

SUMMARY

1. Measurements have been made of the DNA content of cells from 29 human uterine tumours before, during and after treatment by intracavitary radium or radio-cobalt.

2. Six cases showed only slight changes in the pattern of DNA values at 3–7 days after the first treatment. Twenty cases showed the "typical" effect of irradiation, as seen at 7 or 14 days after the first treatment: there was an accumulation of cells having large amounts of DNA, which usually were multiples of the basic DNA value (2, 4 or 8 times). The remaining cases presented certain unusual features.

3. The changes that have been observed have been briefly discussed in relation to the effect of ionizing radiations on DNA synthesis and the mitotic process.

4. While the "typical" response, with the appearance of cells having high DNA values, was seen in tumours which responded well to radiotherapy, 2 tumours which responded poorly were characterized by the appearance of a new modal DNA value in the triploid region. These 2 tumours, which histologically were adenoacanthomata, were also characterized by an unusual pattern of DNA values *before* treatment. The possibility that changes in the DNA content of cells during the early stages of radiotherapy, or perhaps the pattern of DNA values found before treatment, might form the basis of an objective test of radiosensitivity is discussed.

Thanks are due to the radiotherapeutic and gynaecological staff of Mount Vernon Hospital who provided material used in this study, and to Miss M. D. Snelling for kindly allowing material from a case treated at the Middlesex Hospital to be included.

Dr. H. B. Fell, F.R.S., and Dr. M. H. F. Wilkins, F.R.S., kindly read the manuscript, and helpful suggestions were made by Dr. L. H. Gray, Dr. Alma Howard and Mr. D. E. A. Jones. The technical assistance of Mr. D. Doxey is acknowledged. Expenses were defrayed by grants from the British Empire Cancer Campaign.

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